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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appellant:	Jen Sheen	Art Unit:	1638
Serial No.:	08/989,881	Examiner:	C. Collins
Filed:	December 12, 1997	Customer No.	21559
Title:	STRESS-PROTECTED TRANSGENIC PLANTS		

BOX APPEAL
Commissioner For Patents
Washington, DC 20231

APPELLANT'S BRIEF ON APPEAL
SUBMITTED PURSUANT TO 37 CFR § 1.192

In support of Appellant's notice of appeal, received by the Patent Office September 27, 2002, in connection with the Examiner's final rejection mailed on March 28, 2002, submitted herewith in triplicate is appellant's brief on appeal.

TABLE OF CONTENTS

TABLE OF CASES

Real Party in Interest

The real parties in interest in this case are the above-captioned appellant, as well as appellant's assignee, the General Hospital Corporation.

Related Appeals and Interferences

There are no currently pending appeals or interferences related to this case.

Status of Claims

Claims 1-7, 24-26, 36-46, 49, and 50 are currently pending

Claims 1-7, 24-26, 36-46, 49, and 50 were rejected in a final office action mailed on March 28, 2002, and are appealed.

Status of Amendments

All prior amendments in this case have been entered. Appellant points out that a Supplemental Amendment is filed herewith. Appellant requests entry of this amendment and consideration of the appeal based on the claims in Appendix B.

Summary of the Invention

Appellant's invention generally features methods and compositions useful for protecting a plant against an environmental stress. In general, the method involves providing a transgenic plant cell that expresses substantially pure DNA encoding a calcium-dependent protein kinase (CDPK) polypeptide that includes a protein kinase

(PK) domain; and growing a transgenic plant from the plant cell, where the DNA is expressed in the transgenic plant, and where the transgenic plant has increased tolerance to an environmental stress compared to a corresponding untransformed plant. In addition, the invention includes DNA encoding a calcium-dependent protein kinase polypeptide consisting essentially of a protein kinase domain that increases the level of tolerance to an environmental stress in a transgenic plant, and cells including this DNA. The invention also features a plant that includes substantially pure DNA encoding a calcium-dependent protein kinase (CDPK) polypeptide that includes a PK domain, where the polypeptide increases the level of tolerance in a plant expressing the polypeptide to an environmental stress. Importantly, the invention provides methods and compositions for engineering of crop plants protected against an environmental stress.

Issues

Three issues are raised on appeal.

The first issue is whether the Examiner erred in rejecting claims 1-7, 24-26, 36-39, and 41-46 based on the written description requirement of 35 U.S.C. § 112, first paragraph.

The second issue is whether the Examiner erred in limiting the scope of appellant's claims to methods for producing transgenic plants that are drought tolerant as a result of overexpressing a transgene encoding a PK domain of AtCDPK1, thereby rejecting claims 1-7, 24-26, 36-46, and 49-50 as lacking enablement under 35 U.S.C. § 112, first paragraph.

And the third issue is whether the Examiner erred in finding claims 36-46 anticipated by Urao *et al.* (Mol. Gen. Genet. 244:331-340, 1994).

Grouping of Claims

Each of the following groups of claims stands alone and is independently patentable: claims 1-7 and 49, which provide methods for protecting a plant against an environmental stress; claims 24-26 and 50, which feature transgenic plants comprising a DNA encoding a calcium-dependent protein kinase that includes a protein kinase domain; and claims 36-46, which provide a DNA encoding a calcium-dependent protein kinase polypeptide consisting essentially of a protein kinase domain that increases the level of tolerance to an environmental stress in a transgenic plant, and cells that includes this DNA. As is discussed in more detail below, the groups present different issues on the questions of written description, enablement, and anticipation and should be considered separately.

Argument

As is clear from the Issues section above, one or more of the pending claims stand rejected on the grounds of written description, enablement, and anticipation. Each of these rejections, as applied in the final Office Action, and appellant's response to these rejections is now presented.

I. Written Description

Claims 1-7, 24-26, 36-39, and 41-46 stand finally rejected, under 35 U.S.C. § 112, first paragraph, on the basis that the specification provides only a single nucleic acid sequence encoding a calcium-dependent protein kinase that functions to protect plants against stress and therefore does not provide an adequate written description of the invention, as currently claimed. In finally rejecting the claims, the Examiner, in essence, asserts that (1) the protein kinase motif is not a structural motif unique to the genus of sequences useful for protecting a plant against an environmental stress, and (2) a representative number of sequences has not been described.

Appellant respectfully traverses this rejection on the basis that the specification, as of its effective filing date, satisfies the written description requirement set forth by the case law and the U.S. Patent & Trademark Office's Written Description Guidelines (the "Guidelines").

> The Guidelines, under the "Genus Analysis" decision tree, states:
> What is a representative number of species depends on whether one of skill in the art would recognize that applicant was in possession of the necessary common attributes or features of the elements possessed by the members of the genus in view of the species disclosed or claimed. (Emphasis added.)

Appellant has satisfied this standard. Appellant's specification explicitly describes to the skilled worker what is claimed, that is, (i) methods for producing plants that are tolerant to an environmental stress (e.g., drought), (ii) genes encoding polypeptides encoding PK domains, and (iii) methods for isolating and identifying DNAs useful for practicing the claimed invention. For example, the inventor, Dr. Jen Sheen, explicitly describes methods for producing stress tolerant plants at pages 10-11 and 36-37 of the

specification. The inventor also describes, for example, at pages 16-22, not only a stress-signaling expression pathway, but also expression constructs useful for generating plants having tolerance to an environmental stress. Moreover, with respect to isolating genes encoding polypeptides that consist essentially of a PK domain, the inventor explicitly describes (see, for example, Fig. 3C) several PK domains, and also explicitly describes to persons skilled in the art how to select PK domains active in stress signaling, for example, by utilizing the disclosed transient maize protoplast system, described, for example, at pages 13-15.

Together, this description provides one skilled in the art with exemplary PK domains and genetic constructs, as well as methods of using kinase domains to engineer plants that are tolerant to an environmental stress. Clearly, the inventor was in possession of the claimed invention at the time the application was filed and provided a written description that readily enables the skilled worker to identify plants and genes falling within the claimed subject matter.

In a further analysis of the written description requirement, the Guidelines provide an example (Example 17), where the specification disclosed rat cDNA sequences only, but claimed a mammalian or human cDNA sequence. The written description requirement was held not to be satisfied in this case, because:

> . . .neither the specification nor the general knowledge of those skilled in the art provide evidence of <u>any partial structure</u> which would be expected to be <u>common</u> to the members of the genus. Moreover, there is <u>post filing evidence</u> that indicates that there is a <u>lack of structural relationship</u> between the rat insulin cDNA sequences and other mammalian insulin cDNA sequences. (Emphasis added.)

Thus, the implication is that had there been at least a partial structure common to members of the genus, or post-filing evidence of a structural relationship between the members of the genus, then the written description requirement would have been satisfied. The Guidelines, citing applicable case law, also state:

> A description of a genus of cDNAs may be achieved by means of recitation of a representative number of cDNAs, defined by nucleotide sequence, falling within the scope of the genus or of a recitation of structural features common to members of the genus, which features constitute a substantial portion of the genus. *Regents of the University of California v. Eli Lilly & Co.*, 119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1998). (Emphasis added.)

The facts of this case fall squarely with the Office's Written Description Guidelines. Again, the present specification discloses that the nucleic acids useful in the methods of the invention include genes encoding polypeptides having an PK domain, a structural feature common to members of the genus. In addition, post-filing evidence, presented by Saijo *et al.* (Exhibits A and B) provides compelling evidence for not only the existence of CDPK genes that confer tolerance to multiple environmental stresses but also signifies that this structural feature is common to members of the genus. Thus, there can be no question that appellant was in possession of the methods of the invention at the time the application was filed, and that one skilled in the art would recognize appellant's disclosure as a description of the invention defined by the present claims.

In short, appellant's specification clearly satisfies the written description requirement, as set forth by the case law, and appellant requests reconsideration and withdrawal of this basis for the § 112 rejection.

II. Enablement

Claims 1-7, 24-26, 36-46, and 49-50 also stand rejected under § 112, first paragraph based on the assertion that the teaching of appellant's specification is not commensurate in scope with the present claims. The rejection essentially turns on the assertions that practicing the claimed method of protecting plants against an environmental stress would require trial and error experimentation, and that it would also require trial and error experimentation to determine whether the genes required in the methods of the invention, when expressed in a plant, would confer increased tolerance to an environmental stress. This rejection should be withdrawn.

As an initial matter, appellant notes that identifying and using genes falling within the scope of the claims is readily achieved without undue experimentation. On this point, appellant directs the Examiner's attention to the accompanying Declaration of Jen Sheen, the sole inventor on this application, filed in this case April 14, 2000. Dr. Sheen points out that, as outlined in the patent application, the PK domain of AtCDPK1 was placed under the control of the 35S promoter (as described in the specification, for example, at pages 16-19). This expression construct was then introduced into wild-type *Arabidopsis* using standard *Agrobacterium*-mediated transformation methods. Dr. Sheen next compared non-transformed wild-type plants with transgenic plants expressing the PK domain for drought tolerance by withholding water for 10 days. As Dr. Sheen points out, wild-type (non-transformed) plants wilted, but transgenic plants constitutively expressing the PK domain of AtCDPK1 failed to wilt and remained healthy. These data provide compelling evidence that transgenic plants constitutively expressing a polypeptide that

includes a PK domain are tolerant to environmental stresses, such as drought, when compared to corresponding non-transformed plants. Given this evidence, there is no reasonable basis for doubting the objective truth of statements found in appellant's specification regarding enablement or predictability of the present invention. *In re Marzocchi*, 439 F.2d 220, 224, 169 U.S.P.Q. 367, 370 (C.C.P.A. 1971).

Furthermore, with respect to isolating additional PK-domain encoding genes useful in the invention, appellant notes that the specification, for example, at pages 16-20, and as depicted in Figs. 3B and 3C, provides clear guidance, using specific examples, that may be utilized for identifying and isolating a variety of DNA sequences encoding polypeptides having PK domains, from a variety of sources. In addition, the specification at pages 22-24, under the heading "Isolation of Regulators of the Stress Signal Transduction Response," provides general guidance on the routine methods known at the time the application was filed for identifying and characterizing the gene sequences required by the claims. Such standard methods described in the specification include: (1) the design and utilization of oligonucleotides for cloning, (2) hybridization cloning methodologies, (3) library screening procedures, and (4) PCR-based amplification cloning strategies. Together these methods, alone or in combination, are effective for isolating and cloning desired DNAs.

Once a gene sequence is isolated, the specification also provides methods for generating expression constructs using the isolated DNAs and testing whether such constructs activate stress-signaling pathways. For example, at pages 24-31 and at pages 36-37, under the heading "Expression Constructs Encoding Regulators of the Stress

Signal Response," the specification provides clear instructions about how to express such sequences in plants, and how to test whether the sequences express a polypeptide that regulates stress signaling. In one particular example, genes encoding a polypeptide having a PK domain may be readily tested for activating stress signaling using appellant's disclosed protoplast transient expression system, as described in the specification, for example, at pages 13-14. In this system, a construct expressing a polypeptide having a PK domain is coexpressed with a reporter construct (e.g., *HVA1-GFP*), and genes expressing PK domains that activate reporter gene expression are quickly identified as positive regulators of stress signaling. Once identified, such genes are then used to confer tolerance to an environmental stress *in planta*, exactly as described for the experiments discussed by Dr. Sheen, in her declaration, for the PK domain of the AtCDPK1 polypeptide. Alternatively, if desired, isolated DNAs may be tested directly in plants to confirm their ability to confer tolerance to an environmental stress.

In addition, appellant notes that all of the tools for expressing these DNA molecules were known when appellant filed the patent application. Exemplary expression vectors, promoters, and terminators are described in the specification, for example, at pages 28-29, and at pages 30-31. Moreover, the specification, for example, at pages 31-36 describes several methods for introducing the vectors into plant cells, and for regenerating transformed plants. Plants expressing these genes may then be selected, for example, by visual examination (e.g., wilting). Indeed, the data provided in Dr. Sheen's declaration provides strong evidence that plants expressing a polypeptide having

a PK domain have an increased tolerance to environmental stress. Given these exemplary teachings and results, appellant's specification cannot be found as failing to enable the claimed invention when the techniques required to practice the invention are disclosed in the specification and available to those skilled in the art. See *In re Wands, 858 F.2d* 731, 740, 8 USPQ2d 1400, 1406; *In re Strahilevitz*, 668 F.2d 1229,1232, 212 U.S.P.Q. 561, 563 (C.C.P.A. 1982).

As evidence on this point, appellant directs the Examiner's attention to the attached publication by Saijo *et al.* (*Biochimica et Biophysica Acta* 1350: 109-114, 1997; provided as Exhibit A) entitled "cDNA cloning and prokaryotic expression of maize calcium-dependent protein kinases," on which the inventor, Dr. Sheen, is a co-author. In this publication, two maize CDPKs were cloned using degenerate oligonucleotide primers corresponding to conserved regions of the calcium-dependent protein kinase (CDPK) family. Given such an example, there is absolutely no reason to believe that appellant's specification does not enable the isolation and identification of additional CDPK genes from any plant without undue experimentation. Indeed, these approaches require only standard application of routine molecular methods. Accordingly, there is no basis for concluding that one skilled in the art, equipped with appellant's sequences (as well as those already known in the literature) and standard methods known in the art, would not be able to isolate a reasonable number of nucleic acid sequences which encode CDPK genes useful in practicing the methods of the present claims.

In addition, on this issue, appellant notes that determining whether a gene encoding a calcium-dependent protein kinase (CDPK) polypeptide that includes a PK domain

provides protection against an environmental stress is easily accomplished, in a straightforward fashion, simply by overexpressing the gene in a transgenic plant, exactly as taught in the specification at page 24-36.

To substantiate this point further, appellant directs the Examiner's attention to the publication of Saijo *et al.* (*Plant Journal* 23: 319-327, 2000; provided as Exhibit B) entitled "Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants." Here appellant notes that Saijo cloned a rice CDPK gene, called OsCDPK7, using the maize cDNA designated ZmCDPK7 identified in Saijo *et al.* (*Biochimica et Biophysica Acta* 1350: 109-114, 1997; provided as Exhibit A). This result provides still further evidence that CDPK genes are readily isolated and identified using standard methods that were known when the application was filed. Moreover, by overexpressing the rice CDPK gene in transgenic rice plants, Saijo showed that the CDPK gene was a positive regulator of cold and salt/drought stresses, exactly as taught in appellant's specification, for example, at page 13 (lines 3-6). These results corroborate appellant's claim that additional gene sequences may be isolated from a variety of sources using standard techniques that were known in the art and that were useful for practicing the methods of the claimed invention. Furthermore, contrary to the conclusions made by the Office that appellant's teaching does not predict "resistance to a large number of diverse stresses," the teachings of appellant's disclosure has also been shown to be effective not only for drought (see Sheen Declaration of April 14, 2000), but also for cold and salt stresses.

These results confirm the specification's teaching that expression of a CDPK gene that encodes a protein kinase domain promotes protection against a number of environmental stresses (for example, drought, heat, salt, cold, etc.). Accordingly, following the specification's teachings, the skilled worker can simply express a CDPK gene in a plant and then challenge the plant with an environmental stress to evaluate the plant's tolerance to the stress. Plants having increased tolerance are easily distinguished from non-tolerant plants, and the screening of such plants having the desired tolerance to an environmental stress is readily accomplished. Such a single-step screening approach does not constitute undue experimentation as is exemplified by Saijo *et al.* (Exhibit B).

Appellant notes that the data of Saijo *et al.* were obtained using methods known at the time appellant's application was filed, and did not involve the use of improved methods that were developed after the filing date of appellant's application. Thus, the post-filing evidence of Saijo et al. corroborates appellant's assertion that the present application enables the claimed methods available at the time the present application was filed. In particular, the CCPA has indicated that post-filing publications may be used to determine the state of the art existing on the date an application is filed and the relative ease of practicing the invention. For example, the PTO can use later appearing art "as evidence of the state of art existing on the filing date of an application." *In re Hogan*, 559 F.2d 595, 605, 194 USPQ 527, 537 (CCPA 1977). Additionally, post-filing date tests using material and computer programs commercially available on the filing date demonstrated the "relative ease" of developing embodiments. *See Bruning v. Hirose*, 161 F.3d 681, 48 USPQ2d 1934 (Fed. Cir. 1998).

Appellant also points out that, to sustain an enablement rejection, the Office has the initial burden to establish a reasonable basis to question the enabling nature of an appellant's specification. Thus, in a case in which the PTO questions the enablement of a claim, the CCPA, in *In re Marzocchi*, 439 F.2d 220, 169 USPQ 367, 369 (CCPA 1971) has stated that:

> a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the first paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support (emphasis added).

The MPEP (§ 2164.04, Eighth Edition, August 2001) further emphasizes the *Marzocchi* standard in stating that:

> it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. Otherwise there would be no need for the appellant to go to the trouble and expense of supporting his presumptively accurate disclosure (emphasis added).

Appellant notes that no scientific evidence currently made of record in this case establishes a basis for doubting the objective truth of the statements found in appellant's specification regarding enablement with respect to identifying genes falling within appellant's claims and determining whether such genes possess stress resistant properties. As is discussed above, appellant's statement that expression of a gene encoding a CDPK polypeptide that includes a PK domain confers tolerance to an environmental stress in plants expressing such a gene is in accordance with the evidence described in the present

specification for the *Arabidopsis* CDPK gene and the post-filing evidence of Saijo *et al.* Moreover, given the evidence described above, the Examiner has provided no evidence or reason for doubting appellant's statement that other genes having the structural features described by appellant would function similarly to confer tolerance to an environmental stress.

In conclusion, the facts in the present case compel withdrawal of the § 112, first paragraph enablement rejection, and appellant requests reconsideration on this issue.

III. Anticipation

Claims 36-46 were rejected under 35 U.S.C. § 102(b) as anticipated by Urao *et al.* (Mol. Gen. Genet. 244: 331-340, 1994). For the following reasons, appellant respectfully disagrees.

Claim 36 and claims 37- 46, which refer directly or indirectly to claim 36, are drawn to a DNA molecule that encodes a CDPK polypeptide consisting essentially of a PK domain, as well as to cells that include such DNA molecules. Urao *et al.* discloses full-length cDNA molecules encoding full-length CDPK polypeptides. Nowhere does Urao describe a DNA molecule encoding a PK domain consisting essentially of itself. Since Urao does not identically describe the claimed subject matter, it cannot anticipate appellant's claims directed to DNA molecules encoding polypeptides consisting essentially of PK domains or cells that include such DNAs. On this basis alone, appellant respectfully requests reconsideration and withdrawal of the § 102(b) rejection.

In the final office action, the Examiner further asserts that "[t]he sequences taught by Urao *et al.* inherently include a DNA molecule that encodes a polypeptide consisting essentially of a PK domain." Appellant respectfully disagrees here too.

A prior art reference anticipates a patent claim if the reference discloses, either expressly or inherently, all of the limitations of the claims. (*See Kalman v. Kimberley-Clark Corp.*, 713 F2d 760, 771, 218 USPQ781, 789 (Fed. Cir. 1983)). The shortcoming of the Urao article, as acknowledged by the Examiner, is that it does not expressly disclose a DNA molecule encoding a polypeptide consisting essentially of a PK domain. The Office has therefore relied on an inherency theory in finding the claims anticipated.

The operation of inherency and anticipation was explained by the Federal Circuit as follows:

> To serve as an anticipation when the reference is silent about the asserted inherent characteristic, such gap in the reference may be filled with recourse to extrinsic evidence. Such evidence must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. *In re Oelrich,* 666 F.2d 578, 581, 212USPQ 323, 326 (CCPA 1981)(quoting *Hansgirg v. Kemmer,* 102 F.d 212, 214, 40 USPQ 665, 667 (CCPA 1939)) [states]:
>
> > Inherency, however may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient. If, however, the disclosure is sufficient to show that the natural result flowing from the operation as taught would result in the performance of the questioned function, it seems to be well settled that the disclosure should be regarded as sufficient.
>
> This modest flexibility in the rule that "anticipation" requires

> that every element of the claims appear in a single reference accommodates situations where the common knowledge of technologists is not recorded in the reference; that is, where technical facts are known to those in the field of the invention, albeit it not known to judges.

Continental Can Co. U.S.A. v. Monsanto Co., 948 F.2d 1264, 1268-1269, 20 USPQ 2d 1746, 1749-1750 (Fed. Cir. 1991).

Appellant first points out that one skilled in the art would neither interpret nor conclude that Urao's disclosure of full-length CDPK molecules was an unequivocal teaching of fragments of CDPK molecules, much less a fragment encoding polypeptides having a specific biological activity, such as that embodied in a PK domain. The mere possibility that Urao's reference to full-length CDPK DNA molecules might be understood by one of skill in the art to disclose a DNA molecule that encodes a CDPK polypeptide consisting essentially of a PK domain, as claimed, is insufficient to show that it is inherently disclosed in the article. Accordingly, because one skilled in the art, even in view of Urao's full-length cDNAs, would not necessarily recognize the function of a fragment of a portion of the cDNA molecule to be disclosed in the Urao article, the Urao article does not either expressly or inherently anticipate the claimed invention. On this basis too, the § 102 rejection should be withdrawn.

CONCLUSION

Appellant respectfully requests that the rejection of claims 1-7, 24-26, 36-46, and 49-50 be reversed. A check for $160.00 for the required appeal fee is enclosed. If there are any additional charges, or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: 25 April 2003

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Appendix A: Claims on Appeal

1. (Twice amended) A method for protecting a plant against an environmental stress, said method comprising the steps of:

(a) providing a transgenic plant cell that expresses substantially pure DNA encoding a calcium-dependent protein kinase (CDPK) polypeptide that includes a protein kinase (PK) domain; and

(b) growing a transgenic plant from said plant cell, wherein said DNA is expressed in said transgenic plant, and wherein said transgenic plant has increased tolerance to an environmental stress compared to a corresponding untransformed plant.

2. The method of claim 1, wherein said environmental stress is dehydration.

3. The method of claim 1, wherein said environmental stress is excess salinity.

4. The method of claim 1, wherein said environmental stress is a temperature stress.

5. The method of claim 1, wherein said plant is protected against multiple stress conditions.

6. (Once amended) The method of claim 1, wherein the expression of said polypeptide activates the expression of a stress-protective protein-encoding gene.

7. (Once amended) The method of claim 1, wherein said DNA is constitutively expressed in said transgenic plant.

24. (Twice amended) A plant comprising substantially pure DNA encoding a calcium-dependent protein kinase (CDPK) polypeptide that includes a PK domain,

wherein said polypeptide increases the level of tolerance, on a plant expressing said polypeptide, to an environmental stress.

25. A seed from a transgenic plant of claim 24.

26. A cell from a transgenic plant of claim 24.

36. (Twice amended) Substantially pure DNA encoding a calcium-dependent protein kinase (CDPK) polypeptide consisting essentially of a PK domain, said polypeptide being capable of increasing the level of tolerance to an environmental stress in a transgenic plant.

37. (Once amended) The DNA of claim 36, wherein said DNA encodes a polypeptide that confers tolerance to dehydration.

38. (Once amended) The DNA of claim 36, wherein said DNA encodes a polypeptide that confers tolerance to salinity.

39. (Once amended) The DNA of claim 36, wherein said DNA encodes a polypeptide that confers tolerance to a temperature stress.

40. The DNA of claim 36, wherein said DNA comprises a nucleic acid sequence substantially identical to the nucleic acid sequence shown in Fig. 5 (SEQ ID NO: 1).

41. The DNA of claim 36, wherein said DNA is operably linked to an expression control region.

42. (Once amended) The DNA of claim 41, wherein said expression control region comprises a promoter.

43. The DNA of claim 42, wherein said promoter is a constitutive promoter.

44. The DNA of claim 43, wherein said promoter is an inducible promoter.

45. A cell which includes the DNA of claim 36.

46. The cell of claim 45, wherein said cell is a plant cell.

49. The method of claim 1, wherein said calcium-dependent protein kinase (CDPK) polypeptide is ATCDPK1 or ATCDPK1a.

50. The method of claim 24, wherein said calcium-dependent protein kinase (CDPK) polypeptide is ATCDPK1 or ATCDPK1a.

Appendix B: Claims as Amended by Supplemental Amendment

1. (Twice amended) A method for protecting a plant against an environmental stress, said method comprising the steps of:

(a) providing a transgenic plant cell that expresses substantially pure DNA encoding a calcium-dependent protein kinase (CDPK) polypeptide that includes a protein kinase (PK) domain; and

(b) growing a transgenic plant from said plant cell, wherein said DNA is expressed in said transgenic plant, and wherein said transgenic plant has increased tolerance to an environmental stress compared to a corresponding untransformed plant.

2. The method of claim 1, wherein said environmental stress is dehydration.

3. The method of claim 1, wherein said environmental stress is excess salinity.

4. The method of claim 1, wherein said environmental stress is a temperature stress.

5. The method of claim 1, wherein said plant is protected against multiple stress conditions.

6. (Once amended) The method of claim 1, wherein the expression of said polypeptide activates the expression of a stress-protective protein-encoding gene.

7. (Once amended) The method of claim 1, wherein said DNA is constitutively expressed in said transgenic plant.

24. (Three times amended) A plant comprising substantially pure DNA encoding a calcium-dependent protein kinase (CDPK) polypeptide that includes a PK domain, wherein said polypeptide increases the level of tolerance, in a plant expressing said polypeptide, to an environmental stress.

25. A seed from a transgenic plant of claim 24.

26. A cell from a transgenic plant of claim 24.

36. (Twice amended) Substantially pure DNA encoding a calcium-dependent protein kinase (CDPK) polypeptide consisting essentially of a PK domain, said polypeptide being capable of increasing the level of tolerance to an environmental stress in a transgenic plant.

37. (Once amended) The DNA of claim 36, wherein said DNA encodes a polypeptide that confers tolerance to dehydration.

38. (Once amended) The DNA of claim 36, wherein said DNA encodes a polypeptide that confers tolerance to salinity.

39. (Once amended) The DNA of claim 36, wherein said DNA encodes a polypeptide that confers tolerance to a temperature stress.

40. The DNA of claim 36, wherein said DNA comprises a nucleic acid sequence substantially identical to the nucleic acid sequence shown in Fig. 5 (SEQ ID NO: 1).

41. The DNA of claim 36, wherein said DNA is operably linked to an expression control region.

42. (Once amended) The DNA of claim 41, wherein said expression control region comprises a promoter.

43. The DNA of claim 42, wherein said promoter is a constitutive promoter.

44. The DNA of claim 43, wherein said promoter is an inducible promoter.

45. A cell which includes the DNA of claim 36.

46. The cell of claim 45, wherein said cell is a plant cell.

49. The method of claim 1, wherein said calcium-dependent protein kinase (CDPK) polypeptide is ATCDPK1 or ATCDPK1a.

50. (Once amended) The plant of claim 24, wherein said calcium-dependent protein kinase (CDPK) polypeptide is ATCDPK1 or ATCDPK1a.

EXHIBIT A

ELSEVIER

Biochimica et Biophysica Acta 1350 (1997) 109–114

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Short sequence-paper

cDNA cloning and prokaryotic expression of maize calcium-dependent protein kinases [1]

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Abstract

Using degenerate oligonucleotide primers corresponding to conserved regions of the calcium-dependent protein kinase (CDPK) family, we carried out a polymerase chain reaction and obtained four distinct partial-length cDNAs from a maize leaf library. We then used these clones as probes for conventional screening and isolated 19 longer clones from another cDNA library of maize seedlings. These clones were classified into four groups based on their DNA cross-hybridization. Two full-length cDNAs, designated as ZmCDPK9 and ZmCDPK7, were sequenced and characterized. The predicted protein of each clone was a typical CDPK with eleven canonical subdomains of protein kinases, and four EF-hand calcium-binding motifs in its N-terminal and C-terminal halves, respectively. The catalytic and regulatory domains were linked by a well-conserved junction domain. The N-terminus of the protein also contained a consensus sequence for an N-myristoylation signal. Northern blot analysis showed that the transcription level of each gene was higher in roots and etiolated leaves than in green leaves. To confirm the calcium dependency of the maize enzymes, the entire coding region of ZmCDPK9 was subcloned into an expression vector so that it was in frame with the vector-encoded peptide tags. A cell-free extract of *Escherichia coli* transformed with the recombinant plasmid exhibited calcium-dependent phosphorylation activity, using casein as a substrate.

Keywords: Protein kinase, calcium-dependent; Calmodulin-like domain; EF-hand motif; Intracellular calcium signaling; (Maize)

In higher plants, as well as in animals and microorganisms, calcium seems to play a pivotal role as a second messenger by regulating many aspects of cellular signaling [1]. One of the possible mechanisms by which calcium acts in plants is via calcium-dependent protein kinases (CDPKs) [2]. CDPKs contain a calcium-binding regulatory domain similar to calmodulin in their C-termini. Despite the potential importance of CDPKs, little is known about their physiological functions. The only description of a complete CDPK cDNA isolated so far from maize is that of a pollen-specific enzyme [3]. Since we have been searching for CDPKs which are involved in the regulatory phosphorylation of maize phosphoenolpyruvate carboxylase [4], we started to systemati-

Abbreviations: CDPK, calcium-dependent protein kinase or calmodulin-like domain protein kinase; PCR, polymerase chain reaction.

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[1] The nucleotide sequence data reported in this paper have been submitted to the DDBJ/EMBL/GenBank databases under the accession numbers, D85039 (ZmCDPK9), D87042(ZmCDPK7), and D87043-D87046(PCR products).

PII S0167-4781(96)00207-2

cally characterize CDPKs in maize. Here, we report the isolation and characterization of novel cDNA clones encoding maize CDPKs.

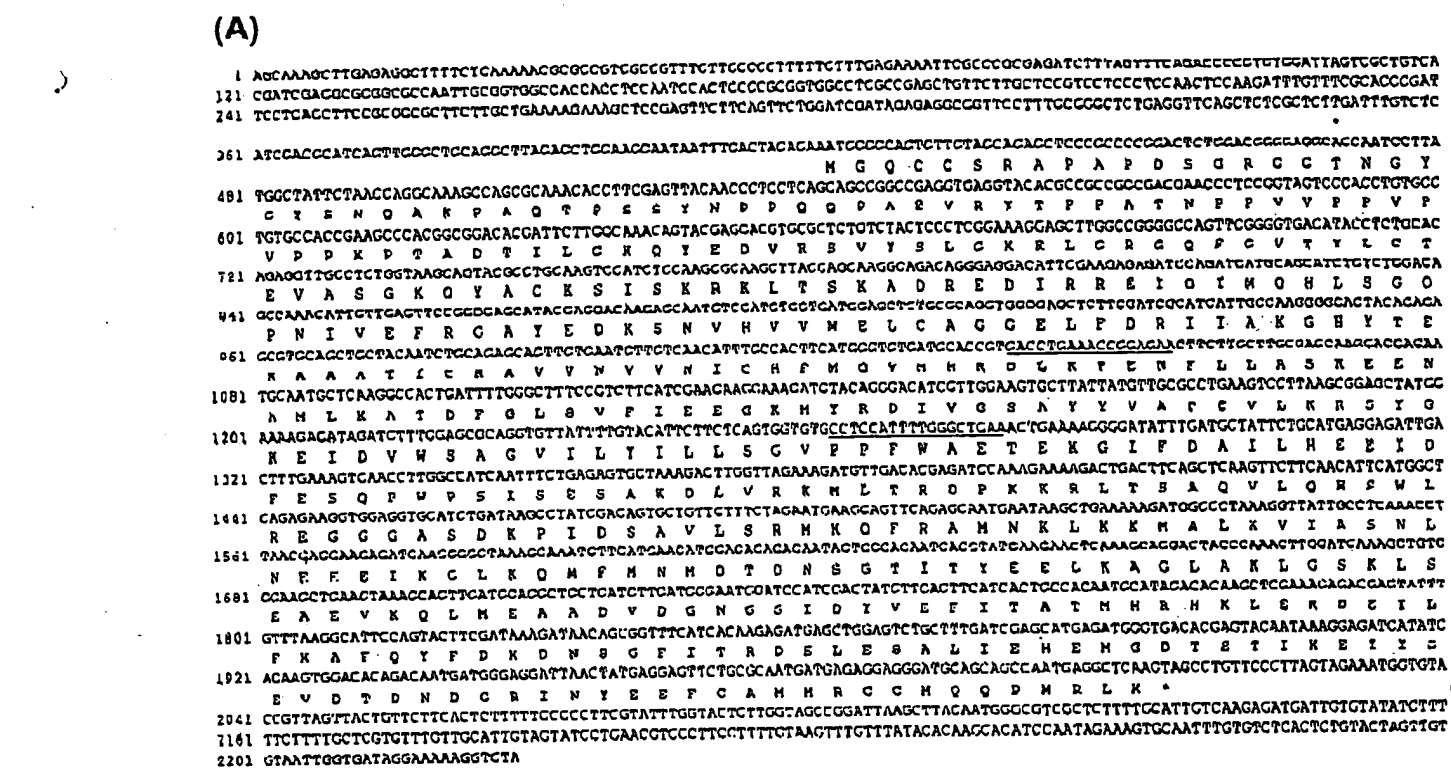
Degenerate oligonucleotide primers were designed for two conserved regions in the catalytic domain of previously cloned CDPKs [3,5–9]. They were 5'-GACCT(G/C)AAGCC(G/T/C)GAGAA-3' and 5'-GCGAATTCGGCCCA(G/A)AA(G/A/T/C)GG-(G/A/T/C)GG-3', corresponding to the sense sequence of DLKPEN and the antisense sequence of PPFWAE, respectively. The former motif is highly conserved in subdomain VIb of serine/threonine kinases, while the latter motif is rather specific to subdomain X of CDPKs (see Fig. 2). After reverse transcription of mRNA from maize (*Zea mays* FR9^{cms} × FR37) green leaves, a polymerase chain reaction (PCR) was performed for 40 cycles (94°C for 30 s, 37°C for 3 min, and 72°C for 30 s) with 20 pM each of the primers [10]. The amplified products (243-263 bp in size) were digested with *Eco*RI, and then subcloned between the *Eco*RI and *Sma*I sites of pBluescript (Stratagene), four putative CDPK clones being thus found (accession numbers, D87043-D87046).

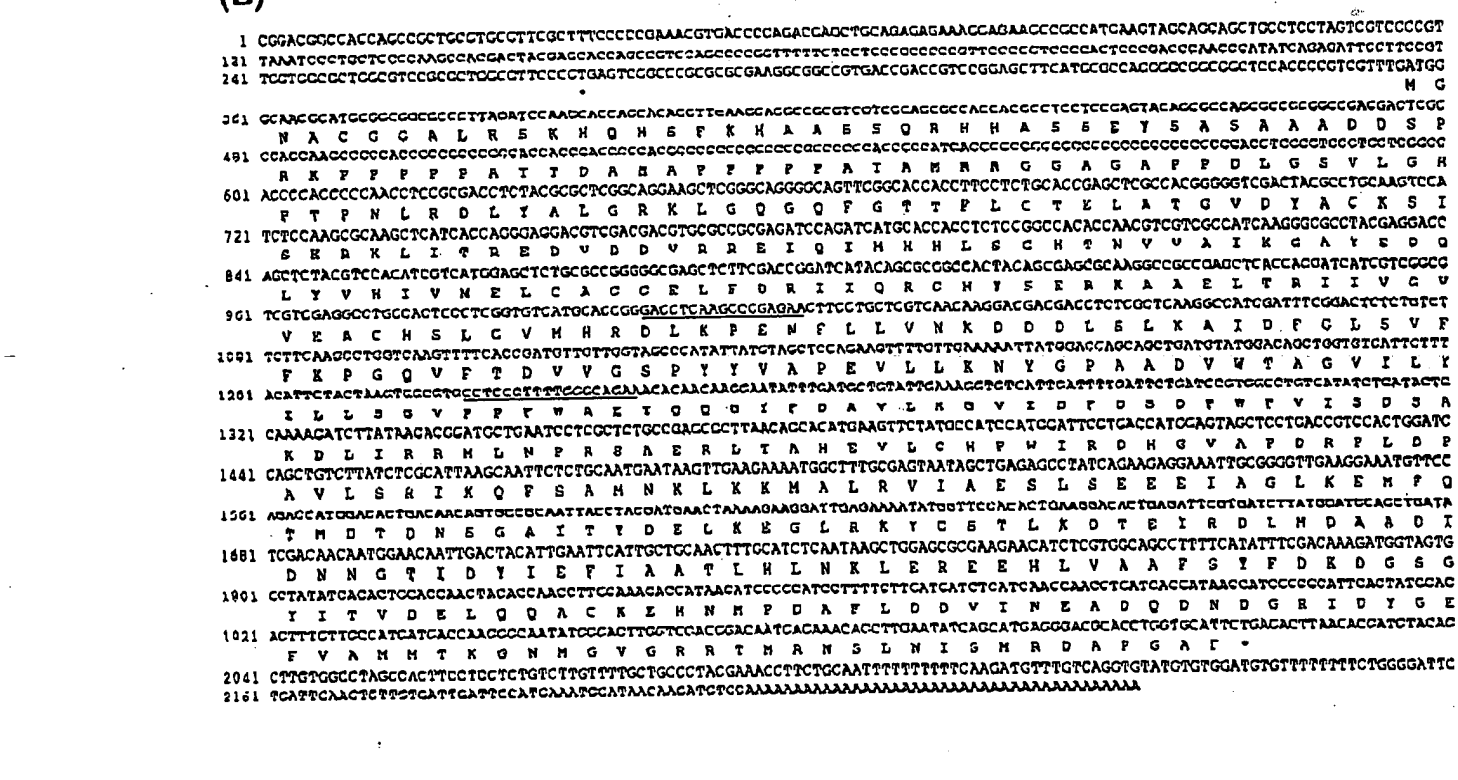
Maize (*Zea mays* H84) seedlings were grown for 7 days at 25°C under a 16-h light/8-h darkness photoperiod. Poly(A)-rich RNA of above-ground portions of the seedlings was used to construct an oligo(dT)-primed cDNA library. Double-stranded cDNA was prepared with a cDNA synthesis kit (Pharmacia), ligated with *Eco*RI/*Not*I adaptors at both ends, and then inserted into the λgt10 vector. Recombinant phages in the resulting maize cDNA library were plated with *Escherichia coli* NM514 and then blotted onto Hybond-N$^+$ membranes (Amersham). Equal amounts of the above-described four putative cDNAs were mixed, and radiolabeled with a Megaprime kit (Amersham) and [α-^{32}P]dCTP. Hybridization and washing were performed as described [11].

Nineteen positive clones were isolated out of 8×10^5 plaques. They were tentatively classified into four distinct groups based on their cross-hybridization. The first and largest group comprised nine clones. The longest cDNA insert (2.3 kb in size) among them was cut out with *Eco*RI, ligated with pBluescript, and then further analyzed. Fig. 1A shows the nucleotide and deduced amino acid sequences of the cDNA clone, ZmCDPK9. An open reading frame for a 531 amino acid polypeptide was found. The predicted molecular mass was 59.4 kDa. Exactly the same sequence as that of one of the four probes (accession no. D87043) was found in ZmCDPK9. However, the probe contained an extra segment of 20 bp. The second group comprised seven clones. The longest cDNA insert (2.2 kb in size) among them was cut out with *Not*I, subcloned into pBluescript, and then sequenced. The clone, designated as ZmCDPK7, has an open reading frame for a 554 amino acid polypeptide, the calculated molecular mass of which is 61.1 kDa (Fig. 1B). The sequence of ZmCDPK7 differed from the corresponding probe (accession no. D87046) by two nucleotides, resulting in one amino acid substitution. These discrepancies are presumed to result from PCR errors or the difference of cultivaters. Characterization of the other two groups is now in progress.

Fig. 2 shows alignment of the amino acid sequences of ZmCDPK9, ZmCDPK7 and other reported CDPKs from maize and rice. ZmCDPK9 had a kinase domain with eleven characteristic subdomains, in its N-terminal half. In the C-terminal half, on the other hand, it had a calmodulin-like domain with four EF-hand calcium binding motifs. A highly conserved junction domain was also found between the kinase and calmodulin-like domains. Thus, ZmCDPK9 encodes a typical CDPK. Similarly, ZmCDPK7 encodes another CDPK. It is noteworthy that both ZmCDPK9 and ZmCDPK7 contained N-terminal amino acid sequences (*MGQCCS* and *MGNACG*, respectively) that fulfill the requirements for a consensus sequence for N-myristoylation [12]. Therefore, the association of the enzymes with the plasma membrane was suggested. A database search revealed a maize CDPK

Fig. 1. Nucleotide and deduced amino acid sequences of ZmCDPK9 (A) and ZmCDPK7(B). Nucleotide positions (beginning at the 5' end) are indicated on the left, and amino acid positions (numbered from the putative translation initiation codon) are indicated on the right. Stop codons at the ends of the open reading frame are denoted by asterisks. Sequences corresponding to the two PCR primers are underlined.





clone, MZECDPK2 (accession no. U28376), which is very similar to ZmCDPK9 (96% identical over 496 amino acids). However, the former does not contain the myristoylation motif, suggesting that the intracellular localization of its protein product is different from that of the latter. It is also noteworthy that the clustered amino acids in the N-terminal portion of the latter differed significantly from those of the former. Another database search did not detect highly homologous maize clones to ZmCDPK7.

The ZmCDPK9 mRNA, 2.3 kb in size, was predominantly expressed in roots (Fig. 3A). In addition, its level was much higher in etiolated leaves than in green ones (Fig. 3A). The ZmCDPK7 mRNA, 2.3 kb in size, was expressed in a similar manner (Fig. 3B). It seems likely that the expression of these transcripts is repressed by light. In this regard, it is noticeable that CDPKs have been partially purified from etiolated coleoptiles and enclosed leaf rolls [13]. At present, however, it remains to be investigated if mRNA expression reflects protein levels in tissue distribution.

To examine the enzyme activity of ZmCDPK9, we cut out the entire cDNA with *Not*I, inserted it into

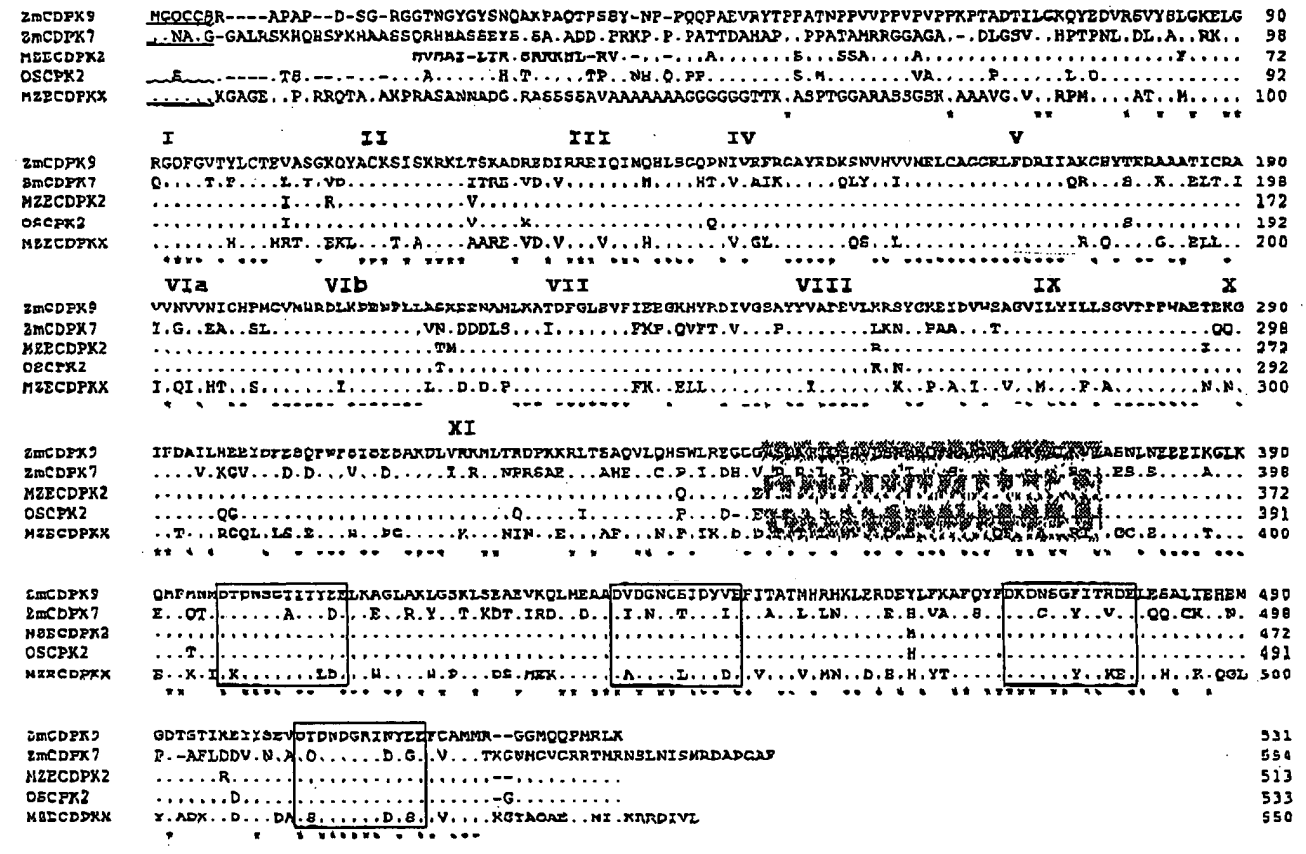


Fig. 2. Comparison of the deduced amino acid sequences of ZmCDPK9, ZmCDPK7, maize CDPKs (MZECDPKX [3] and MZECDPK2 (accession no. U28376)), and a rice CDPK (OsCPK2 [9]). Numbers indicate amino acid residues in the sequences. Dots indicate residues identical to the corresponding ones in the ZmCDPK9 sequence. Asterisks indicate residues identical throughout. Dashes indicate gaps introduced to maximize the alignment. Roman numerals indicate the eleven canonical subdomains of protein kinases identified by Hanks and Hunter [19]. The junction domain is shaded. The consensus N-myristoylation signal is underlined, and the four Ca^{2+}-binding EF-hand motifs are boxed.

Fig. 3. Northern blot analyses of the ZmCDPK9 (A) and ZmCDPK7 (B) transcripts. Each lane contained 30 μg of total RNA isolated from different tissues of 10-day-old maize seedlings: green leaves (lane G), etiolated leaves (E), and roots (R). The RNAs were subjected to electrophoresis in a denaturing 1.2% agarose gel, and then transferred to Hybond-N$^+$ membranes. The membranes were probed with the ^{32}P-labeled 0.2 kb *Hin*dIII fragment containing the 3′-noncoding region of ZmCDPK9 or a 0.2 kb PCR fragment corresponding to nucleotides 2020-2205 of ZmCDPK7. To confirm the integrity of each RNA preparation, one of the membranes was reprobed with a cDNA for *Glycine max* ubiquitin [20] (C). The size of the detected band was estimated using a poly(A)-tailed RNA ladder (Gibco BRL) as molecular size markers.

the *Not*I site of pET32a (Novagen), and then removed 5′-noncoding region as follows. Two oligonucleotides, 5′-GACTAGAGCCATGGGGCAGTGTTG-3′ and 5′-TGTACAAAATAACACCTGCGCTCC-3′, were synthesized. The former was specific for the N-terminus of ZmCDPK9 open reading frame and contained an artificial *Nco*I site to facilitate the construction. The latter, on the other hand, was specific for the sequence corresponding to nucleotides 1218-1241 which is downstream of a natural AflII site. The PCR was carried out with the ZmCDPK9 plasmid as a template. The resulting product was digested with *Nco*I and AflII, and then cloned between the *Nco*I and AflII sites of the pET construct. Partial sequencing of the expression plasmid, designated as pETPK9, showed a correct frame for a chimeric protein of the Trx · tag, His · tag and S · tag (160 amino acids) and the entire protein kinase (531 amino acids). *E. coli* BL21 cells transformed with

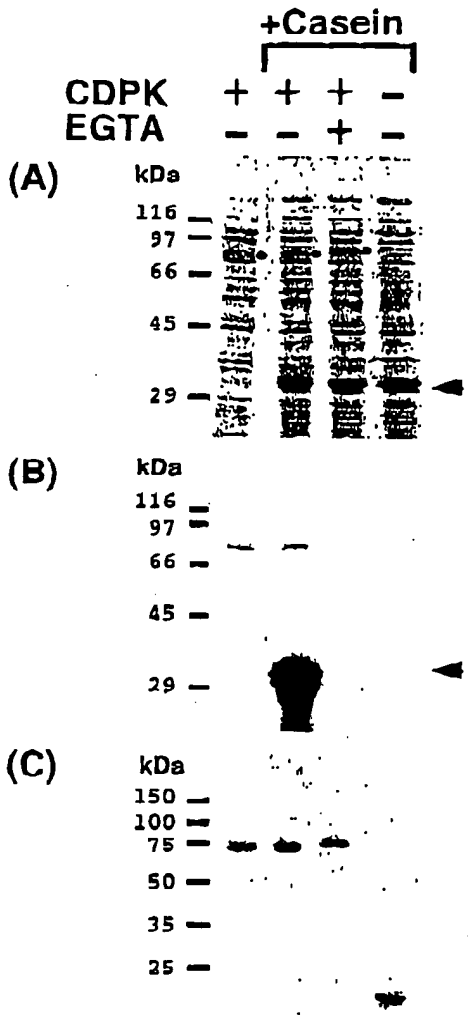


Fig. 4. Prokaryotic expression of the phosphorylation activity of ZmCDPK9 by the recombinant plasmid, pETPK9. Cell-free extracts of *E. coli* transformed with pETPK9 (CDPK+) or pET32a vector (CDPK−) were incubated with [γ-^{32}P]ATP at 25°C for 30 min with (+) or without (−) 1 mM EGTA as described in the text. The reaction products were divided into two portions and then subjected to 10% SDS-PAGE. After the electrophoresis, one gel was stained with Coomassie Blue (A) and then subjected to a Bio-imaging analyzer for determination of radioactivity (B). The proteins on the other gel were transferred to a polyvinylidenedifluoride membrane (Millipore). The membrane was probed with S-proteinalkaline phosphatase conjugate (Novagen) and the colour was developed according to the manufacturer (C). The positions of ZmCDPK9 fusion protein and casein in the gel are indicated by dots and arrowheads, respectively. The positions and sizes of molecular weight markers (A and B, Sigma; and C, Novagen) are presented in kDa on the left.

pETPK9 were grown in LB medium containing 50 μg/ml ampicillin at 37°C. An overnight culture of *E. coli* was diluted 10-fold and grown for an additional 3 h at 37°C before addition of 1.0 mM (in final concentration) isopropyl-1-thio-β-galactopyranoside and then it was grown for an additional 25 h at 25°C. Extraction of the enzyme was performed essentially as described [14,15]. The protein concentration was determined by the method of Bradford [16]. The reaction mixture (final volume, 20 μl) comprised 20 μM [γ-^{32}P]ATP (4 μCi), 50 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 20 μg casein (Sigma), and the enzyme (20 μg protein). It was incubated at 25°C for 30 min, and separated by 10% SDS-PAGE, and then the radioactivity was determined with a Bio-imaging analyzer (Fuji).

As shown in Fig. 4B, a cell-free extract of *E. coli* containing the recombinant chimeric protein efficiently phosphorylated casein, an exogenous substrate. The expression of fusion protein was confirmed by Western blotting (Fig. 4C). Notably, the enzyme activity was completely inhibited by an excess amount of EGTA. It is also noteworthy that the mobility of ZmCDPK fusion protein in the SDS-polyacrylamide gel was altered by the presence of EGTA (Fig. 4A and C). This Ca^{2+}-dependent mobility shift, generally regarded as a characteristic of high-affinity Ca^{2+}-binding proteins, indicated that the ZmCDPK9 protein binds Ca^{2+} [17,18]. Thus, it was proved that ZmCDPK9 encodes a typical CDPK. The upper bands in Fig. 4B might result from autophosphorylation of the expressed fusion protein. Phosphoenolpyruvate carboxylase from maize was a poor substrate for this CDPK (data not shown). It is important to clarify the physiological role of the protein kinase in the future.

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References

[1] Bush, D.S. (1995) Annu. Rev. Plant Physiol. Plant Mol. Biol. 46, 95–122.
[2] Roberts, D.M. and Harmon, A.C. (1992) Annu. Rev. Plant Physiol. Plant Mol. Biol. 43, 375–414.
[3] Estruch, J.J., Kadwell, S., Merlin, E. and Crossland, L. (1994) Proc. Natl. Acad. Sci. USA 91, 8837–8841.
[4] Izui, K., Yabuta, N., Ogawa, N., Ueno, Y., Furumoto, T., Saijo, Y., Hata, S. and Sheen, J. (1995) in Photosynthesis: from Light to Biosphere (Mathis, P., ed.), Vol. 5, pp. 163–166. Kluwer, Dordrecht.
[5] Harper, J.F., Sussman, M.R., Schaller, G.E., Putnam-Evans, C., Charbonneau, H. and Harmon, A.C. (1991) Science 252, 951–954.
[6] Suen, K.-L. and Choi, J.H. (1991) Plant Mol. Biol. 17, 581–590.
[7] Harper, J.F., Binder, B.M. and Sussman, M.R. (1993) Biochemistry 32, 3282–3290.
[8] Urao, T., Katagiri, T., Mizoguchi, T., Yamaguchi-Shinozaki, K., Hayashida, N. and Shinozaki, K. (1994) Mol. Gen. Genet. 244, 331–340.
[9] Breviario, D., Morello, L. and Giani, S. (1995) Plant Mol. Biol. 27, 953–967.
[10] Sheen, J. (1993) EMBO J. 12, 3497–3505.
[11] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
[12] Johnson, D.R., Bhatnagar, R.S., Knoll, L.J. and Gordon, J.I. (1994) Annu. Rev. Biochem. 63, 869–914.
[13] Battey, N.H. (1990) Biochem. Biophys. Res. Commun. 170, 17–22.
[14] Inoue, T., Osumi, T. and Hata, S. (1995) Biochim. Biophys. Acta 1260, 49–54.
[15] Nakashima, T., Inoue, T., Oka, A., Nishino, T., Osumi, T. and Hata, S. (1995) Proc. Natl. Acad. Sci. USA 92, 2328–2332.
[16] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[17] Burgess, W.H., Jemiolo, D.K. and Kretsinger, R.H. (1980) Biochim. Biophys. Acta 623, 257–270.
[18] Ling, V. and Zielinski, R.E. (1993) Plant Mol. Biol. 22, 207–214.
[19] Hanks, S.K. and Hunter, T. (1995) FASEB J. 9, 576–596.
[20] Fortin, M.G., Purohit, S.K. and Verma, D.P.S. (1988) Nucleic Acids Res. 16, 11377.

EXHIBIT B

The Plant Journal (2000) 23(3), 319–327

Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants

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Summary

A rice gene encoding a calcium-dependent protein kinase (CDPK), OsCDPK7, was induced by cold and salt stresses. To elucidate the physiological function of OsCDPK7, we generated transgenic rice plants with altered levels of the protein. The extent of tolerance to cold and salt/drought stresses of these plants correlated well with the level of OsCDPK7 expression. Therefore, OsCDPK7 was shown to be a positive regulator commonly involved in the tolerance to both stresses in rice. Over-expression of OsCDPK7 enhanced induction of some stress-responsive genes in response to salinity/drought, but not to cold. Thus, it was suggested that the downstream pathways leading to the cold and salt/drought tolerance are different from each other. It seems likely that at least two distinct pathways commonly use a single CDPK, maintaining the signalling specificity through unknown post-translational regulation mechanisms. These results demonstrate that simple manipulation of CDPK activity has great potential with regard to plant improvement.

Keywords: CDPK, cold, salt, drought, rice, stress tolerance.

Introduction

Environmental stresses, such as cold, salinity and drought, have an enormous impact on crop productivity throughout the world (Boyer, 1982; Epstein *et al.*, 1980). To survive under unfavourable conditions, plants have developed a variety of sophisticated strategies (Bohnert *et al.*, 1995; Bray, 1997; Thomashow, 1999; Zhu *et al.*, 1997). The products of some stress-inducible genes (e.g. enzymes involved in the biosynthesis of various osmoprotectants and late-embryogenesis-abundant (LEA) proteins) directly counteract the detrimental conditions. Transfer of these genes into plants confirmed their protective roles in stress adaptation (Holmberg and Bulow, 1998; Xu *et al.*, 1996). Although the effect of each individual gene is rather small, simultaneous transcriptional activation of a subset of these genes can confer much greater stress tolerance on *Arabidopsis* (Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998). It is generally thought that distinct mechanisms underlie the adaptation to cold and salt/drought stresses in plants (Shinozaki and Yamaguchi-Shinozaki, 1997; Zhu *et al.*, 1997). Both appear to be regulated by complex signalling networks of abscisic acid (ABA)-dependent pathways and/or ABA-independent pathways (Ishitani *et al.*, 1997; Leung and Giraudat, 1998; Shinozaki and Yamaguchi-Shinozaki, 1997). It has been predicted that the modulation of signalling regulators will be a promising method for improving the stress tolerance of plants. However, little is known about the molecular mechanisms underlying signal transduction.

Previous studies showed that the cytoplasmic Ca^{2+} levels in plant cells increase rapidly in response to multiple stress stimuli, including cold, salt and drought (Sanders *et al.*, 1999; Trewavas and Malho, 1997). Following this Ca^{2+} influx, signals are likely to be mediated by combinations of protein phosphorylation/dephosphorylation cascades. The experimental perturbation with specific reagents indicated the pivotal roles of Ca^{2+} influx and protein phosphorylation in these stress responses (Knight *et al.*, 1996; Knight *et al.*, 1997; Monroy and Dhindsa, 1995; Monroy *et al.*, 1993; Monroy *et al.*, 1998; Tahtiharju *et al.*, 1997). It is presumed that the majority of Ca^{2+}-stimulated

protein phosphorylation is performed predominantly by members of the Ca^{2+}-dependent protein kinase (CDPK) family in plants (Sanders *et al.*, 1999; Trewavas and Malho, 1997). This kinase family, currently only known in plants and protozoa, contains a calmodulin-like regulatory domain with four EF hands, Ca^{2+}-binding site, at its C-terminal end, which enables activation directly through Ca^{2+} binding (Roberts and Harmon, 1992). Two of the four constitutively active mutant enzymes of two related *Arabidopsis* CDPKs activated a stress/ABA-responsive promoter in a transient expression system, indicating that selected members of the CDPK family are involved in that particular stress signalling (Sheen, 1996). In addition, stress-induced gene expression of some CDPK members has been reported in various plant species, and they are proposed to mediate stress signals (Berberich and Kusano, 1997; Botella *et al.*, 1996; Urao *et al.*, 1994; Yoon *et al.*, 1999). However, it is not known whether or not the increases in mRNA levels are accompanied by increases in protein levels and/or kinase activities. It is also noteworthy that a number of transport proteins (e.g. aquaporins, H^+-ATPases and ion channels), which are responsible for cytosolic osmoregulation and involved in stress adaptation, are regulated by CDPKs (Bethke and Jones, 1997; Camoni *et al.*, 1998a; Li *et al.*, 1998; Lino *et al.*, 1998; Pei *et al.*, 1996; Weaver and Roberts, 1992). Despite the potential importance of CDPKs, the physiological function of a specific CDPK pathway has not been elucidated so far.

In the current study, we investigated the function of a rice cold- and salt-inducible CDPK, OsCDPK7, by using transgenic rice plants with altered levels of the protein. Over-expression of OsCDPK7 conferred both cold and salt/drought tolerance on rice plants. In contrast, suppression of OsCDPK7 expression lowered the stress tolerance. The results indicate that OsCDPK7 plays key roles in the tolerance to both stresses in rice.

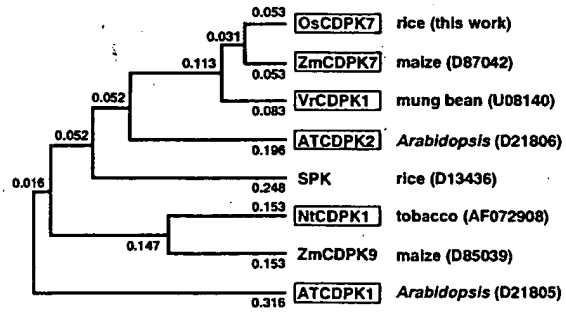

Figure 1. Phylogenetic tree for the amino acid sequences of CDPKs. The UPGMA method (Nei, 1987) was applied using Genetyx-Mac software (Software Development, Tokyo, Japan). The values on the branches give branch length as the number of amino acid substitutions per site. CDPKs, the transcripts of which are stress-inducible, are boxed. Plant sources and GenBank accession numbers are indicated.

Results

cDNA cloning of OsCDPK7

Two maize CDPKs, ZmCDPK1 and ZmCDPK7, showed 97.1% identity at the amino acid level over the entire polypeptide (Berberich and Kusano, 1997; Saijo *et al.*, 1997), and the mRNA expression of both CDPK genes were induced by cold stress (Berberich and Kusano, 1997; data not shown). Using the *ZmCDPK7* cDNA as a probe, we isolated a highly homologous rice clone, designated *OsCDPK7* (GenBank accession no. AB042550), from a rice root cDNA library (Hata *et al.*, 1997). In contrast to the maize genome, Southern blot analysis of rice genomic DNA with the 3′-non-coding region (nt 1815–2061) as a probe indicated that the rice genome contains only a single copy of this gene (data not shown).

OsCDPK7 and ZmCDPK7 showed 88.6% identity at the amino acid level over the entire polypeptide. In addition, OsCDPK7 showed high amino acid identity to a mung bean CDPK, VrCDPK1 (85.4%), the transcript level of which was elevated by salt stress and mechanical strain (Botella *et al.*, 1996). They were classified into the same subclass of CDPKs in a phylogenetic tree for the amino acid sequences of the entire ORFs (Figure 1). A further database search revealed an *Arabidopsis* expressed sequence tag (EST) clone (accession no. R90026) encoding a portion of CDPK (124 amino acids). The deduced protein sequence was more closely related to OsCDPK7 than ATCDPK1 (Sheen, 1996; Urao *et al.*, 1994), a putative *Arabidopsis* stress-signalling isoform (amino acid identities of 80.6 and 52.8%, respectively). Therefore, it seems likely that OsCDPK7, ZmCDPK7, ZmCDPK1, VrCDPK1 and the *Arabidopsis* protein may be orthologues that play identical roles under stress conditions.

The entire coding region of OsCDPK7 was subcloned into an expression vector, pGEX4T-1 (Amersham Pharmacia), and then transformed into *Eschericia coli* BL21 (DE3). Protein kinase activity was assayed as described (Saijo *et al.*, 1997). The recombinant OsCDPK7 protein fused with glutathione-*S*-transferase (GST) efficiently phophorylated histone IIIS, casein, and myelin basic protein (Sigma) in a Ca^{2+}-dependent manner (data not shown). This broad substrate specificity is in sharp contrast to the narrow specificity of a GST–ATCDPK1 fusion protein (Urao *et al.*, 1994), indicating that OsCDPK7 and ATCDPK1 differ in substrate preferences.

OsCDPK7 *is induced by cold and salt stresses*

Expression of the *OsCDPK7* mRNA, around 2.3 kb in size, was increased by cold and salt stresses in both shoots (Figure 2a) and roots (data not shown) of 10-day-old seedlings, but not by exogenous abscisic acid (ABA) application. In contrast, a rice stress-responsive gene,

rab16A (Mundy and Chua, 1988), was induced by the salt and ABA treatments (Figure 2a). This indicates that OsCDPK7 belongs to a subclass of stress-inducible CDPKs, which is probably conserved from monocotyledonous to dicotyledonous plants.

To determine whether or not the protein level increases upon stressing, we then carried out immunoblot analyses with isoform-specific antibodies raised against the C-terminal portion of OsCDPK7. A clear band corresponding to an apparent molecular mass of 51 kDa was seen in the soluble fractions of the protein extracts from plant tissues (Figure 2b). Significant variation in the protein level was not detected during the cold-stress period examined in either the shoots (Figure 2b) or roots (data not shown), even when the mRNA accumulated to a high level (Figure 2a). The protein levels were almost similar between shoots and roots (data not shown). In the microsomal membrane fractions precipitated by the centrifugation, no signal for the OsCDPK7 protein was detected (data not shown). Similar results were obtained under salt stress (data not shown). Thus, the intracellular localization of the enzyme protein is as yet unclear. Moreover, the shoot proteins immunoprecipitated with anti-OsCDPK7 antibodies did not exhibit any changes in kinase activity (data not shown). However, the activity seemed to be affected by contaminated Ca^{2+} during the preparation of protein extracts. Therefore, in this experiment, additional factor(s) that positively or negatively influence the activity other than Ca^{2+} could not be detected either.

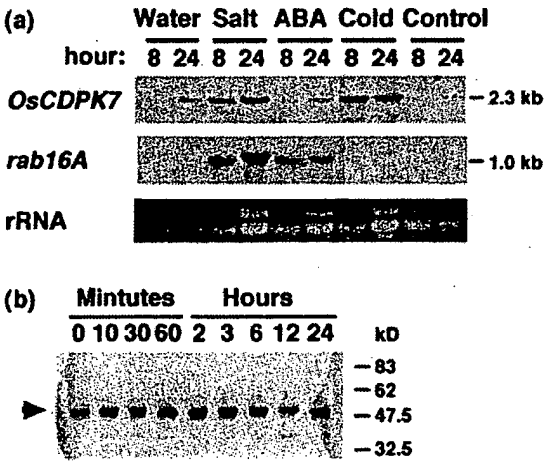

Figure 2. Expression of *OsCDPK7* in wild-type rice plants.
(a) Cold and salt induction of the *OsCDPK7* transcript. Ten-day-old rice (*Oryza sativa* cv. Notohikari) seedlings in soil were left to stand under the normal conditions at 28°C (control) or at 4°C (cold) for the indicated times. Alternatively, they were transferred to nutrient solution comprising 0.1% (v/v) Hyponex (water) supplemented with 200 mM NaCl (salt) or 50 μM ABA (ABA), and then incubated at 28°C for the indicated times. These treatments were conducted under continuous light conditions. Total RNA preparations (10 μg lane^{-1}) from shoots were loaded. The RNA blot was sequentially hybridized with the *OsCDPK7*-and *rab16A*-specific DNA probes.
(b) OsCDPK7 protein levels. Soluble protein extracts were prepared from shoots of 10-day-old seedlings which had been incubated at 4°C for the indicated times. The extracts (10 μg protein lane^{-1}) were separated by 10% SDS-polyacrylamide gel electrophoresis, and then subjected to immunoblot analysis with anti-OsCDPK7 antibodies. An arrowhead indicates the position of OsCDPK7.

Transgenic rice plants with altered expression levels of OsCDPK7

To clarify the physiological role of OsCDPK7 in rice, the full-length cDNA (nt 1–1970) for OsCDPK7 was introduced into rice cells in the sense orientation under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Figure 3a) by means of *Agrobacterium*-mediated transformation (Hiei *et al.*, 1994). Then, 14 independent lines of transgenic plants (T0 generation) were generated. DNA blot analysis of these lines confirmed that independent lines contained one to several copies of the transgene per haploid genome (data not shown). Among them, two lines over-expressing *OsCDPK7*, S3 and S1, and another co-suppressed line, S27, were chosen for further experiments. In the next T1 generation, the levels of the OsCDPK7 protein were constitutively higher in the two over-expressing lines, S3 and S1, than those in the segregated non-transgenic (NT) line derived from S3 in both roots and shoots (Figure 3b). No obvious effects on plant growth and development were observed on OsCDPK7 over-expression under the normal growth conditions. On the other hand, the protein level in S27 was much lower than that in NT (Figure 3b). Since the protein levels were similar between homozygous and heterozygous plants in each line (data

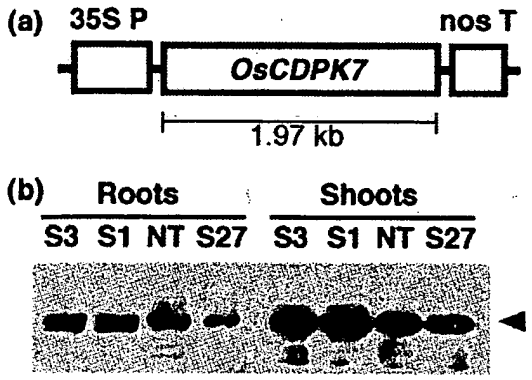

Figure 3. Transgenic rice plants with altered protein levels of OsCDPK7.
(a) The OsCDPK7 expression vector for rice transformation. 35S P, CaMV 35S promoter; *OsCDPK7*, the full-length cDNA (1.97 kb in size) for OsCDPK7; nos T, terminator sequence of the gene for nopaline synthase.
(b) Expression of the OsCDPK7 protein in transgenic and non-transgenic rice plants. NT, a segregated non-transgenic line derived from S3. Each lane contained 5 μg (roots) or 10 μg (shoots) protein from 10-day-old T1 seedlings grown under the normal conditions. The protein blots were probed with anti-OsCDPK7 antibodies. An arrowhead indicates the position of OsCDPK7.

not shown), both types of T1 transformants were combined and then used for further analyses. Significant variations in the protein level in each line were not detected even under cold- and salt-stress conditions (data not shown), when the transcript levels were increased (also see Figure 5).

Stress tolerance of transgenic OsCDPK7 *rice plants*

To examine cold tolerance, 10-day-old T1 seedlings were exposed to 4°C for 24 h, and then returned to the normal growth conditions to allow their recovery. The extent of cold tolerance correlated well with the level of OsCDPK7 expression (Figure 4a). The elevated tolerance of the OsCDPK7-over-expressing plants was confirmed by measuring the changes in the chlorophyll fluorescence yield in the youngest extended leaf of each plant. The F_v/F_m values recovered to nearly normal levels in S3 and S1 plants 48 h after cold treatment (Figure 4b). In contrast, the values progressively decreased in the segregated NT plants derived from S3, their leaves showing prominent chlorosis and wilting. In another independent experiment, similar results were obtained (Table 1).

Next, the over-expressing plants also showed an increased tolerance to salt stress (Figure 4c). In more than half of the NT-S3 plants and untransformed wild-type plants, the youngest leaves wilted 3 days after salt stress. On the other hand, S3 and S1 plants exhibited greater tolerance (statistically significant) (Table 1). In addition, 13-day-old T2 seedlings of S1 plants showed increased drought tolerance with statistical significance (Figure 4d and Table 1). All the NT plants wilted 5 days after drought stress, whereas more than half of the S1 plants did not.

To determine whether or not a decrease in OsCDPK7 expression lowers the stress tolerance, we compared the number of plants whose leaves wilted under milder conditions between the OsCDPK7-suppressed plants (S27) and the segregated NT plants derived from S27. In

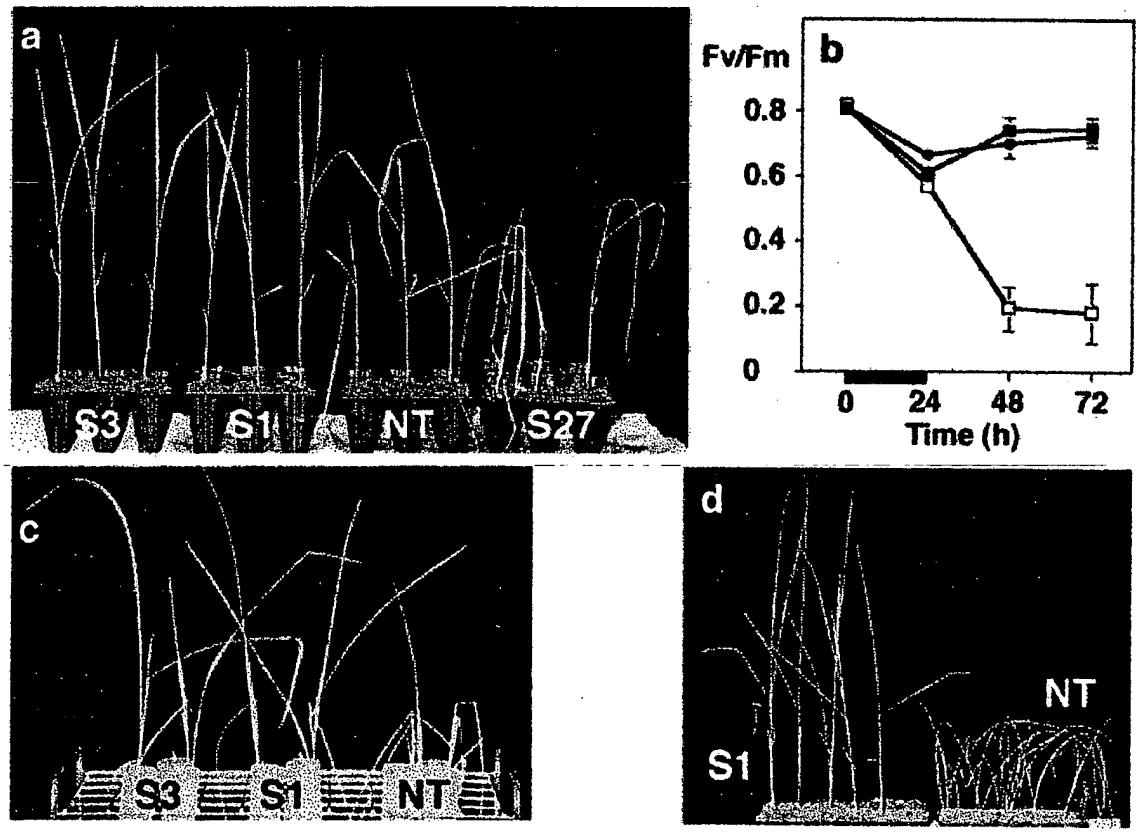

Figure 4. Stress tolerance of 35S:*OsCDPK7* transgenic rice plants.
(a) Plants 3 days after cold stress (4°C for 24 h). (b) Changes in the chlorophyll fluorescence of the youngest extended leaf under cold stress. Each point shows the mean and standard error of F_v/F_m values, from which functional damage to photosynthesis can be estimated. (■) S3; (●) S1; (□) NT. For the S3, S1, and NT lines, 25, 21 and 9 plants, respectively, were assayed. The bar below the *x* axis represents the period of cold-stress treatment. (c) Plants 3 days after salt stress (200 mM NaCl for 24 h). (d) Plants 5 days after drought stress (without water supply for 3 days).

S27 plants, the tolerance to both of these stresses was significantly decreased as follows. After milder cold stress, wilting was observed in 18/33 (55%) S27 plants and 3/9 (33%) NT plants. After milder salt stress, the wilting ratios of S27 and NT plants were 9/35 (26%) and 0/12 (0%), respectively. Taken together, we conclude that OsCDPK7 is a positive regulator commonly involved in the adaptation to at least three distinct stress agents, cold, salt and drought.

Expression of stress-inducible genes in transgenic OsCDPK7 *plants*

The induction of numerous stress-responsive genes is a hallmark of stress adaptation in plants (Shinozaki and Yamaguchi-Shinozaki, 1997; Thomashow, 1999; Zhu *et al.*, 1997). To elucidate further the role of OsCDPK7 in stress tolerance, we examined the effects of OsCDPK7 over-expression on the transcript levels of several stress-inducible rice genes – *rab16A* (Mundy and Chua, 1988), *salT* (Claes *et al.*, 1990) and *wsi18* (Takahashi *et al.*, 1994) – that encode a group 2 late-embryogenesis-abundant (LEA) protein, a glycine-rich protein, and a group 3 LEA protein, respectively. Since it is thought that similar mechanisms underlie the adaptation to water deficit caused by drought and high salinity (Bray, 1997; Zhu *et al.*, 1997), we analysed the expression of these genes in roots only under cold and salt stresses.

In both roots and shoots of the OsCDPK7-over-expressing plants (S3 and S1), the *OsCDPK7* transcript was accumulated at high concentrations. The level was increased further under both cold and salt stresses, whereas it remained almost constant in the presence of exogeneous ABA or under drought stress conditions (Figure 5). Notably, OsCDPK7 over-expression did not induce the above stress-inducible genes under the normal growth conditions (Figure 5), in contrast to resukts obtained for the *CBF1*-and *DREB1A*-over-expressing *Arabidopsis* plants (Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998). In roots of the over-expressing plants under salt stress, the transcript levels of all the stress-inducible genes were higher than the normally induced levels (Figure 5a). In shoots, only *rab16A* was highly induced by both salt and drought stress (Figure 5b,c). In contrast, salt induction was reduced in the suppressed plants (Figure 5a,b). The salt/drought stress tolerance of the over-expressing plants may be enhanced, at least in part, by the high-level accumulation of these

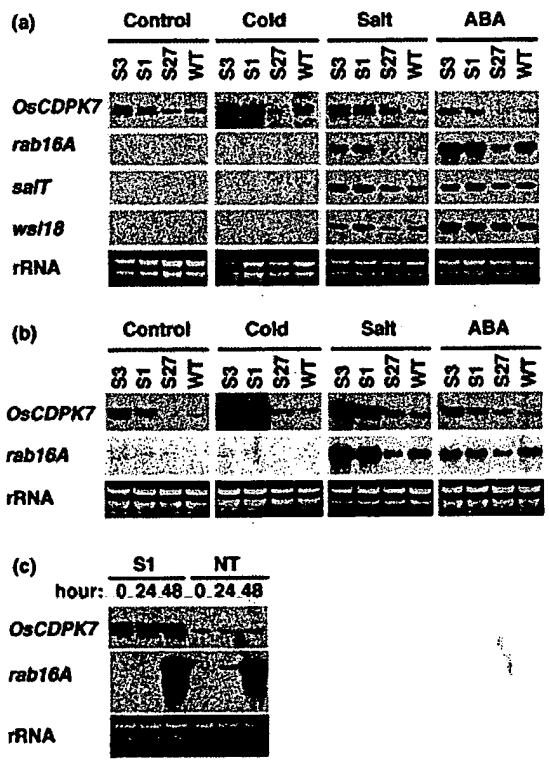

Figure 5. OsCDPK7 activates distinct signalling pathways in response to cold and salt/drought stresses.
(a,b) Northern analyses of RNAs of roots (a) and shoots (b) of wild-type (WT) and transgenic T1 plants. Ten-day-old seedlings were exposed to the following stress conditions. Control, the normal conditions; cold, 4°C for 24 h; salt, soaking in a 200 mM NaCl solution for 10 h (a) or 24 h (b); ABA, soaking in a 50 μM ABA solution for 24 h. (c) Northern analysis on transgenic T2 plants exposed to drought stress. Thirteen-day-old seedlings of the plants homozygous for the transgene (S1) and those of NT plants had water withheld for the indicated times. For each treatment, at least 7 plants per line were examined. Aliquots (10 μg) of total RNA were loaded on each lane. The RNA blots were hybridized with the specific DNA probes for *OsCDPK7*, *rab16A*, *salT* and *wsi18*. Experiments were repeated three times with similar results.

Table 1. Cold, salt and drought stress tolerance of the wild-type (WT) and transgenic *OsCDPK7* rice plants

Stress	Genotype	Total	Wilting (%)
Cold[a]	S3[d]	20	0 (0)
	S1[d]	17	2 (12)
	NT-S3	6	5 (83)
Salt[b]	S3[d]	21	3 (14)
	S1[d]	12	0 (0)
	NT-S3	7	4 (57)
	WT	7	4 (57)
Drought[c]	S1[d]	17	8 (47)
	NT-S1	24	24 (100)

[a]Numbers of WT and T1 plants, the youngest leaves of which wilted 3 days after cold stress (4°C for 24 h). [b]Numbers of WT and T1 plants, the youngest leaves of which wilted 3 days after salt stress (200 mM NaCl for 24 h). [c]Numbers of wilting T2 plants 5 days after drought stress (without water supply for 3 days). [d]Genotypes showing a statistically significant difference from the NT plants (χ^2 test, $P < 0.005$).

gene products through the OsCDPK7 pathway. On the other hand, no induction of these genes was detected under cold stress. Thus, it was suggested that mechanisms of cold tolerance and salt/drought tolerance are different from each other, sharing OsCDPK7 as a common component. Moreover, it should be noted that the induction of *rab16A* and *salT* by ABA was increased or decreased by OsCDPK7 over-expression or suppression, respectively, which indicates that OsCDPK7 is also involved in ABA-dependent pathways.

In addition to the above-mentioned genes, the salt/ABA induction but not cold induction of *rab16B* (Yamaguchi-Shinozaki *et al.*, 1989) and *oslea3* (Moons *et al.*, 1997), both of which encode LEA proteins, was found to be increased in roots of the over-expressing plants (data not shown). The expression of the following rice cold-responsive genes was also examined: *alcohol dehydrogenase-1* (Xie and Wu, 1989), *lip5*, *lip9'*, and *lip19* (Aguan *et al.*, 1991), Δ^1-*pyrroline-5-carboxylate synthetase* (Igarashi *et al.*, 1997) and *glutathione reductase-2* (Kaminaka *et al.*, 1998). However, they exhibited no correlated induction with the OsCDPK7 expression levels (data not shown). Thus, we have not yet identified genes induced only by cold stress. Nevertheless, it still remains possible that currently unknown genes are regulated by the OsCDPK7 pathways under cold stress.

Discussion

Considering its importance as the most major crop in the world, a better understanding of stress signalling in rice would undoubtedly have an enormous impact. Here we characterized a rice CDPK, OsCDPK7, the mRNA level of which is increased under cold- and salt-stress conditions in both shoots and roots. Database searches revealed the presence of genes encoding this type of stress-inducible CDPK in both monocots and dicots. OsCDPK7 belongs to a subclass of stress-inducible CDPKs, which is conserved throughout higher plants but distinct from that of ATCDPK1, a putative stress-signalling isoform (Sheen, 1996; Urao *et al.*, 1994). The difference in substrate specificity also indicated that OsCDPK7 is involved in other signalling pathway(s) than that of ATCDPK1 under stress conditions.

Immunoblot analysis of the protein level of OsCDPK7 in rice plants with the isoform-specific antibodies detected no significant increase under the stress conditions, even when the mRNA level was elevated. There are other examples of putative stress-signalling mitogen-activated protein kinases (MAPKs) in plants in which transcriptional up-regulation is not correlated with an increase in the amount of protein (Bogre *et al.*, 1997; Seo *et al.*, 1999). It may be speculated that the activated MAPK is degraded immediately after it has transduced signal(s), and the transcriptional up-regulation of the MAPK gene is to compensate for the loss of the MAPK protein (Hirt, 1999). To know whether or not this is the case for OsCDPK7, it is important to examine turnover of the protein under the stress conditions. Since the OsCDPK7 protein is expressed at an almost constant level in the presence or absence of stress stimuli, there must be a post-translational mechanism(s) regulating the kinase activity in plant cells. At present, Ca^{2+} is the only known regulator of the activity of CDPKs. However, it seems likely that additional unknown mechanism(s) may be involved in control of the OsCDPK7 activity under stress conditions, taking into account the broad substrate specificity of this kinase. In this regard, recent studies suggested that some of the isoforms might be regulated by interaction with other proteins, e.g. 14-3-3 proteins (Camoni *et al.*, 1998b; Moorhead *et al.*, 1999). Even though neither activation of the OsCDPK7 enzyme upon stress nor protein–protein interaction was detected, the possibility that such regulation mechanisms are involved *in vivo* cannot be ruled out at present. Moreover, it is still possible that changes in protein localization could be important in the regulation of OsCDPK7.

We then carried out a functional analysis of the role of OsCDPK7 in stress tolerance of rice plants, using transgenic plants with altered levels of the OsCDPK7 protein. The mRNA derived from the endogenous promoter and that derived from the CaMV 35S promoter might differ in their stability, because the transgene is truncated in the 3'-non-coding region. However, it seems likely that the same intact protein is translated from both types of the mRNA, since Western blot analyses detected a single band in each lane of the over-expressing plants (Figure 3b). Over-expression of OsCDPK7 conferred both cold and salt/drought tolerance on rice plants. In contrast, suppression of OsCDPK7 expression lowered the stress tolerance. Therefore, OsCDPK7 plays key roles in the tolerance to the two types of stress in rice. To our knowledge, this is the first demonstration of the physiological role of a CDPK isoform at the whole-plant level.

The enhanced salt/drought induction of the genes for LEA proteins by OsCDPK7 over-expression appeared to contribute, at least in part, to the improved salt/drought tolerance in rice plants. Notably, the over-expression of a barley (*Hordeum vulgare* L.) group 3 LEA protein, HVA1, conferred both salt and drought stress tolerance on transgenic rice plants (Xu *et al.*, 1996). Although OsCDPK7 over-expression also elevated the ABA-induced levels of the above genes, whether or not the salt induction of these genes depends on ABA remains to be examined.

In contrast to salt/drought stress, no induction of these genes was observed under cold stress or in the absence of a stress stimulus. Although little is known about the OsCDPK7-mediated cold signalling pathway, we suggest that OsCDPK7 promotes cold and salt/drought tolerance

through distinct pathways. Moreover, it seems likely that OsCDPK7 is kept normally inactive, since there is no constitutive induction of the above stress-inducible genes upon OsCDPK7 over-expression. Thus, OsCDPK7 over-production only is insufficient to trigger the downstream signalling, and stress stimuli must be required to activate this CDPK. Consistent with this speculation, no significant effects were observed with regard to development, growth and fertility in over-expressing plants grown in a greenhouse (unpublished results). This is very favourable for crop improvement. For instance, *DREB1A*-over-expressing plants showed severe growth retardation under normal growth conditions, presumably because of the constitutive high-level expression of stress-inducible genes (Liu *et al.*, 1998). In contrast to this transcription factor, it appears that the activity of OsCDPK7 is under a stringent post-translational control in rice cells.

Finally, we propose a model for the OsCDPK7 signalling pathway under the above stress conditions, in which the amount of activated OsCDPK7 determines the transduction current (Figure 6). The results also suggest that OsCDPK7 acts at one of the branch points of stress signal transduction in rice. Nevertheless, there seems little or no cross-talk downstream of these CDPK pathways even when each signal is amplified by OsCDPK7 over-expression. There must be unknown mechanisms that maintain the signalling specificity. Future analyses, especially on protein–protein interactions and protein localization, are awaited to verify this model. The present work provides new approaches for engineering of crop plants with improved stress tolerance, as well as for understanding the principles governing CDPK-mediated stress signal transduction.

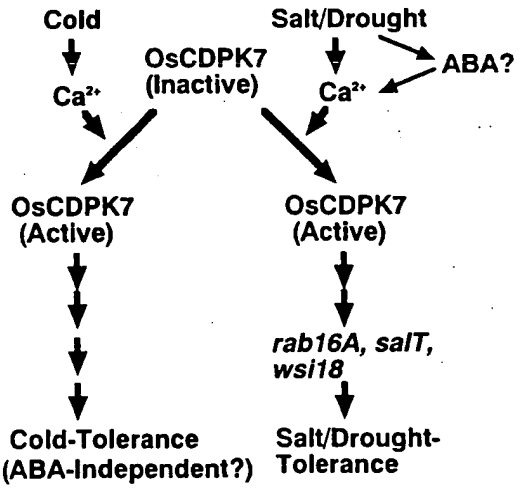

Figure 6. A model for OsCDPK7-mediated cold-and salt/drought-stress signal transduction.

Experimental procedures

cDNA cloning

Using a cDNA for ZmCDPK7 as a probe, cDNA cloning of *OsCDPK7* was carried out as described by Saijo *et al.* (1997) from a rice (*Oryza sativa* Nipponbare) root cDNA library (Hata *et al.*, 1997). Two positive clones were isolated, and both of them were confirmed to encode OsCDPK7 by sequencing. Clones 1 and 2 correspond to nt 1–1970 and nt 453–2126, respectively, of the composite cDNA sequence for OsCDPK7.

Northern blot analysis

Total RNA was prepared from plant tissues according to Clark (1997). Northern blot analysis was performed as described by Saijo *et al.* (1997) using the following probes: the 3′-non-coding region of *OsCDPK7* (nt 1815–2061), and portions of *rab16A* (nt 669–899) (Mundy and Chua, 1988), *salT* (nt 290–690) (Claes *et al.*, 1990) and *wsi18* (nt 678–933) (Takahashi *et al.*, 1994).

Production of anti-OsCDPK7 antibodies

To prepare anti-OsCDPK7 antibodies, a cDNA fragment encoding the C-terminal portion (amino acid residues 449–551) of OsCDPK7 was subcloned into the pET32a vector (Novagen), and then transformed into *E. coli* BL21 (DE3). The expressed recombinant protein was purified on a histidine tag affinity column (Novagen), and then the peptide tags on the N-terminal side were removed by enterokinase digestion. Polyclonal rabbit antibodies raised against the antigen were purified using a column of Sepharose 4B (Amersham-Pharmacia) coupled with the GST–OsCDPK7 fusion protein. The purified antibodies did not cross-react with another CDPK isoform, ZmCDPK9, extracted from *E. coli* (BL21) cells harbouring pETPK9 (Saijo *et al.*, 1997).

Preparation of protein extracts and immunoblot analysis

Plant tissues were ground in liquid nitrogen and then homogenized in an extraction buffer (50 mM Tris–HCl (pH 7.6), 2 mM EDTA, 1 mM $MgCl_2$, 2 mM DTT, 1 mM NaF, 10 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 1 mM phenylmethanesulphonyl fluoride, one tablet per 50 ml protease inhibitor cocktail (Boehringer), 10% (v/v) polyvinylpolypyrrolidone). The homogenate was centrifuged first at 10 000 *g* for 10 min, and then at 100 000 *g* for 45 min. The supernatant was transferred into clean tubes, immediately frozen in liquid nitrogen, and then stored at −80°C. For immunoblot analysis, the supernatant was desalted by passing it through a Sephadex G-25 column (Amersham-Pharmacia) equilibrated with 50 mM Tris–HCl (pH 7.6), containing 1 mM DTT. Western blot analysis was performed essentially as described by Ueno *et al.* (2000) with anti-OsCDPK7 antibodies. The signals were then detected by an enhanced chemiluminescence system (Boehringer).

Rice transformation

A full-length cDNA clone (nt. 1–1970) for OsCDPK7 was introduced into a Ti-based vector, pMSH1 (Kawasaki *et al.*, 1999), in the sense orientation downstream of the CaMV 35S promoter. The construct was introduced into rice calli (*Oryza sativa* cultivar Notohikari) by means of *Agrobacterium*-mediated transformation, according to the published protocol (Hiei *et al.*, 1994).

Transformed calli were selected for hygromycin resistance, and then transgenic plants were regenerated.

Estimation of stress tolerance of transgenic rice plants

In a growth chamber (16 h light/8 h darkness, 28°C), sterilized seeds were germinated on Murashige and Skoog agar medium (Murashige and Skoog, 1962) for 5 days, and then transplanted in soil. For cold-stress treatment, 10-day-old seedlings were exposed to 4°C for 24 h under continuous light conditions, and then returned to the normal growth conditions. Stress treatments under milder conditions were conducted as follows. Ten-day-old seedlings were first incubated at 15°C for 24 h, then at 4°C for 24 h, and finally returned to the normal conditions. For salt-stress treatment, 10-day-old seedlings were transferred to a nutrient solution, 0.1% (v/v) Hyponex (Hyponex Japan), containing 200 mM NaCl or 150 mM NaCl for the milder treatments, and then incubated for 24 h under the normal light/dark cycle at 28°C. Immediately after the salt stress, the roots of the plants were rinsed with water, and then hydroponically grown in a fresh nutrient solution without NaCl. Finally, in order to determine whether or not each T1 plant has the transgene, we amplified a portion of *OsCDPK7* (nt 661–945) from the genomic DNA of each plant by means of PCR, using 5′-ACATCGTCATGGAGCTCTGCGCC-3′ and 5′GAGCTACGTAATATGGGCTTCCG-3′ as primers. A 285 bp fragment without intron sequences was amplified from each transgenic plant. The plants of each T1 line examined included both the homozygous and heterozygous plants. For drought-stress treatment, 13-day-old T2 seedlings of the plants homozygous for the transgene (S1) and those of non-transgenic plants (NT) had water withheld for 3 days, and were then irrigated normally for 5 days.

Determination of chlorophyll fluorescence

Measurement of chlorophyll fluorescence was performed with a pulse-amplitude modulation fluorometer (PAM-2000; Walz, Effeltrich, Germany). Fluorescence signals from the youngest extended leaf of each rice plant, which had been dark-adapted for 15 min, were measured at the indicated times. The ratio of F_v to F_m (F_v/F_m) representing the activity of photosystem II was used to assess the functional damage to the plants (Genty *et al.*, 1989).

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References

Aguan, K., Sugawara, K., Suzuki, N. and Kusano, T. (1991) Isolation of genes for low-temperature-induced proteins in rice by a simple subtractive method. *Plant Cell Physiol.* **32**, 1285–1289.

Berberich, T. and Kusano, T. (1997) Cycloheximide induces a subset of low temperature-inducible genes in maize. *Mol. Gen. Genet.* **254**, 275–283.

Bethke, P.C. and Jones, R.L. (1997) Reversible protein phosphorylation regulates the activity of the slow-vacuolar ion channel. *Plant J.* **11**, 1227–1235.

Bogre, L., Ligterink, W., Meskiene, I., Barker, P.J., Heberle-Bors, E., Huskisson, N.S. and Hirt, H. (1997) Wounding induces the rapid and transient activation of a specific MAP kinase pathway. *Plant Cell*, **9**, 75–83.

Bohnert, H.J., Nelson, D.E. and Jensen, R.G. (1995) Adaptations to environmental stresses. *Plant Cell*, **7**, 1099–1111.

Botella, J.R., Arteca, J.M., Somodevilla, M. and Arteca, R.N. (1996) Calcium-dependent protein kinase gene expression in response to physical and chemical stimuli in mungbean (*Vigna radiata*). *Plant Mol. Biol.* **30**, 1129–1137.

Boyer, J.S. (1982) Plant productivity and environment. *Science*, **218**, 443–448.

Bray, E.A. (1997) Plant responses to water deficit. *Trends Plant Sci.* **2**, 48–54.

Camoni, L., Fullone, M.R., Marra, M. and Aducci, P. (1998a) The plasma membrane H^+-ATPase from maize roots is phosphorylated in the C-terminal domain by a calcium-dependent protein kinase. *Physiol. Plant*, **104**, 549–555.

Camoni, L., Harper, J.F. and Palmgren, M.G. (1998b) 14-3-3 proteins activate a plant calcium-dependent protein kinase (CDPK). *FEBS Lett.* **430**, 381–384.

Claes, B., Dekeyser, R., Villarroel, R., Van den Bulcke, M., Bauw, G., Van Montagu, M. and Caplan, A. (1990) Characterization of a rice gene showing organ-specific expression in response to salt stress and drought. *Plant Cell*, **2**, 19–27.

Clark, S.E. (ed.) (1997) *Plant Molecular Biology – A Laboratory Manual.* Berlin: Springer-Verlag.

Epstein, E., Norlyn, J.D., Rush, D.W., Kingsbury, R.W., Kelley, D.B., Cunningham, G.A. and Wrona, A.F. (1980). Saline culture of crops: a genetic approach. *Science*, **210**, 399–404.

Genty, B., Briantais, J.-M. and Baker, N.R. (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochim. Biophys. Acta*, **990**, 87–92.

Hata, S., Sanmiya, K., Kouchi, H., Matsuoka, M., Yamamoto, N. and Izui, K. (1997) cDNA cloning of squalene synthase genes from mono- and dicotyledonous plants, and expression of the gene in rice. *Plant Cell Physiol.* **38**, 1409–1413.

Hiei, Y., Ohta, S., Komari, T. and Kumashiro, T. (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. *Plant J.* **6**, 271–282.

Hirt, H. (1999) Transcriptional upregulation of signaling pathways: more complex than anticipated? *Trends Plant Sci.* **4**, 7–8.

Holmberg, N. and Bulow, L. (1998) Improving stress tolerance in plants by gene transfer. *Trends Plant Sci.* **3**, 61–66.

Igarashi, Y., Yoshida, Y., Sanada, Y., Yamaguchi-Shinozaki, K., Wada, K. and Shinozaki, K. (1997) Characterization of the gene for Δ^1-pyrroline-5-carboxylate synthetase and correlation between the expression of the gene and salt tolerance in *Oryza sativa* L. *Plant Mol. Biol.* **33**, 857–865.

Ishitani, M., Xiong, L., Stevenson, B. and Zhu, J.-K. (1997) Genetic analysis of osmotic and cold stress signal transduction in Arabidopsis: interactions and convergence of abscisic acid-dependent and abscisic acid-independent pathways. *Plant Cell*, **9**, 1935–1949.

Jaglo-Ottosen, K.R., Gilmour, S.J., Zarka, D.G., Schabenberger, O. and Thomashow, M.F. (1998) *Arabidopsis CBF1* overexpression

induces *COR* genes and enhances freezing tolerance. *Science*, **280**, 104–106.

Kaminaka, H., Morita, S., Nakajima, M., Masumura, T. and Tanaka, K. (1998) Gene cloning and expression of cytosolic glutathione reductase in rice (*Oryza sativa* L.). *Plant Cell Physiol.* **39**, 1269–1280.

Kawasaki, T., Henmi, K., Ono, E., Hatakeyama, S., Iwano, M., Satoh, H. and Shimamoto, K. (1999) The small GTP-binding protein Rac is a regulator of cell death in plants. *Proc. Natl Acad. Sci. USA*, **96**, 10922–10926.

Knight, H., Trewavas, A.J. and Knight, M.R. (1996) Cold calcium signaling in Arabidopsis involves two cellular pools and a change in calcium signature after acclimation. *Plant Cell*, **8**, 489–503.

Knight, H., Trewavas, A.J. and Knight, M.R. (1997) Calcium signaling in *Arabidopsis thaliana* responding to drought and salinity. *Plant J.* **12**, 1067–1078.

Leung, J. and Giraudat, J. (1998) Abscisic acid signal transduction. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **49**, 199–222.

Li, J., Lee, Y.R. and Assmann, S.M. (1998) Guard cells possess a calcium-dependent protein kinase that phosphorylates the KAT1 potassium channel. *Plant Physiol.* **116**, 785–795.

Lino, B., Baizabal-Aguirre, V.M. and Gonzalez de la Vara, L.E. (1998) The plasma membrane H^+-ATPase from beet root is inhibited by a calcium-dependent phosphorylation. *Planta*, **204**, 352–359.

Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. *Plant Cell*, **10**, 1391–1406.

Monroy, A.F. and Dhindsa, R.S. (1995) Low-temperature signal transduction: induction of cold acclimation-specific genes of alfalfa by calcium at 25°C. *Plant Cell*, **7**, 321–331.

Monroy, A.F., Sarhan, F. and Dhindsa, R.S. (1993) Cold-induced changes in freezing tolerance, protein phosphorylation, and gene expression. Evidence for a role of calcium. *Plant Physiol.* **102**, 1227–1235.

Monroy, A.F., Sangwan, V. and Dhindsa, R.S. (1998) Low temperature signal transduction during cold acclimation: protein phosphatase 2A as an early target for cold-inactivation. *Plant J.* **13**, 653–660.

Moons, A., Keyser, A.D. and Van Montagu, M. (1997) A group 3 LEA cDNA of rice, responsive to abscisic acid, but not to jasmonic acid, shows variety-specific differences in salt stress response. *Gene*, **191**, 197–204.

Moorhead, G., Douglas, P., Cotelle, V. *et al.* (1999) Phosphorylation-dependent interactions between enzymes of plant metabolism and 14-3-3 proteins. *Plant J.* **18**, 1–12.

Mundy, J. and Chua, N.-H. (1988) Abscisic acid and water-stress induce the expression of a novel rice gene. *EMBO J.* **7**, 2279–2286.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473–497.

Nei, M. (1987) *Molecular Evolutionary Genetics*. New York: Columbia University Press.

Pei, Z.M., Ward, J.M., Harper, J.F. and Schroeder, J.I. (1996) A novel chloride channel in *Vicia faba* guard cell vacuoles activated by the serine/threonine kinase, CDPK. *EMBO J.* **15**, 6564–6574.

GenBank database accession number AB042550.

Roberts, D.M. and Harmon, A.C. (1992) Calcium-modulated proteins: targets of intracellular calcium signals in higher plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43**, 375–414.

Saijo, Y., Hata, S., Sheen, J. and Izui, K. (1997) cDNA cloning and prokaryotic expression of maize calcium-dependent protein kinases. *Biochim. Biophys. Acta*, **1350**, 109–114.

Sanders, D., Brownlee, C. and Harper, J.F. (1999) Communicating with calcium. *Plant Cell*, **11**, 691–706.

Seo, S., Sano, H. and Ohashi, Y. (1999) Jasmonate-based wounding signal transduction requires activation of WIPK, a tobacco mitogen-activated protein kinase. *Plant Cell*, **11**, 289–298.

Sheen, J. (1996) Ca^{2+}-dependent protein kinases and stress signal transduction in plants. *Science*, **274**, 1900–1902.

Shinozaki, K. and Yamaguchi-Shinozaki, K. (1997) Gene expression and signal transduction in water-stress response. *Plant Physiol.* **115**, 327–334.

Tahtiharju, S., Sangwan, V., Monroy, A.F., Dhindsa, R.S. and Borg, M. (1997) The induction of *kin* genes in cold-acclimating *Arabidopsis thaliana*. Evidence of a role for calcium. *Planta*, **203**, 442–447.

Takahashi, R., Joshee, N. and Kitagawa, Y. (1994) Induction of chilling resistance by water stress, and cDNA sequence analysis and expression of water stress-regulated gene in rice. *Plant Mol. Biol.* **26**, 339–352.

Thomashow, M.F. (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50**, 571–599.

Trewavas, A.J. and Malho, R. (1997) Signal perception and transduction: the origin of the phenotype. *Plant Cell*, **9**, 1181–1195.

Ueno, Y., Imanari, E., Emura, J. *et al.* (2000) Immunological analysis of the phosphorylation state of maize C_4-form phosphoenolpyruvate carboxylase with specific antibodies raised against a synthetic phosphorylated peptide. *Plant J.* **21**, 17–26.

Urao, T., Katagiri, T., Mizoguchi, T., Yamaguchi-Shinozaki, K., Hayashida, N. and Shinozaki, K. (1994) Two genes that encode Ca^{2+}-dependent protein kinase are induced by drought and high-salt stresses in *Arabidopsis thaliana*. *Mol. Gen. Genet.* **244**, 331–340.

Weaver, C.D. and Roberts, D.M. (1992) Determination of the site of phosphorylation of Nodulin 26 by the calcium-dependent protein kinase from soybean nodules. *Biochemistry*, **31**, 8954–8959.

Xie, Y. and Wu, R. (1989) Rice alcohol dehydrogenase genes: anaerobic induction, organ specific expression and characterization of cDNA clones. *Plant Mol. Biol.* **13**, 53–68.

Xu, D., Duan, X., Wang, B., Hong, B., Ho, T.-H.D. and Wu, R. (1996) Expression of a late embryogenesis abundant protein gene, *HVA1*, from barley confers tolerance to water deficit and salt stress in transgenic rice. *Plant Physiol.* **110**, 249–257.

Yamaguchi-Shinozaki, K., Mundy, J. and Chua, N.H. (1989) Four tightly linked *rab* genes are differentially expressed in rice. *Plant Mol. Biol.* **14**, 29–39.

Yoon, G.M., Cho, H.J., Ha, H.J., Liu, J.R. and Lee, H.-S.P. (1999) Characterization of *NtCDPK1*, a calcium-dependent protein kinase gene in *Nicotiana tabacum*, and the activity of its encoded protein. *Plant Mol. Biol.* **39**, 991–1001.

Zhu, J.K., Hasegawa, P.M. and Bressan, R.A. (1997) Molecular aspects of osmotic stress in plants. *Crit. Rev. Plant Sci.* **16**, 253–277.